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The role of conserved aspartate and serine residues in ligand binding and in function of the 5-HT_{1A} receptor: a site-directed mutation study

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Wild-type and mutant serotonin 1A receptors were transiently expressed in COS-7 cells using the infection–transfection variant of the vaccinia virus/T7 polymerase vector system. The amino acid substitutions in the transmembrane regions, Asp^{82}→Asn^{82}, Asp^{116} →Asn^{116}, and Ser^{198}→Ala^{198} all resulted in a decrease in affinity for 5-HT by 60–100-fold, without affecting the affinity for the antagonist, pindolol. The binding of agonist to the additional mutant, Thr^{199}→Ala^{199}, was too weak to be measured. 5-HT induced GTPase activities for all receptors studied. These findings indicate that the residues mutated play an important role in the binding of the agonist and less critical roles in the binding of the antagonist pindolol.

Serotonin 5-HT_{1A} receptor; Vaccinia virus vector; Receptor affinity; GTPase; COS-7 cell

1. INTRODUCTION

The serotonin 1A receptor (5-HT_{1A}R) is a member of the 7-helix, G protein-activating receptor family [1,2]. Recently the 5-HT_{1A}R was expressed in a variety of mammalian cell types [3] using the vaccinia virus (VV)/T7 polymerase vector system; in cardiac atrial cells, the expressed receptor activated K^+ channels within 1 s [4]. Endogenous 5-HT_{1A}R also activates K^+ channels in hippocampal neurons without the involvement of known soluble second messengers [5], a property of the 5-HT_{1A}R which may be relevant for its moderately rapid neurotransmitter signaling (i.e. 100 ms–1 s). Both findings are consistent with the report that in isolated patches, these channels can be gated directly by GTP-activated $G\alpha_i$ and/or $G\alpha_o$ subunits [6].

The present study extends the VV/T7 polymerase vector system to experiments on site-directed mutations for the 7-helix receptor family. We examine the effects of several amino acid residues on agonist and antagonist binding, and on activation of G proteins. β-Adrenergic receptor (β-AR) agonists, such as norepinephrine, share with 5-HT the feature of a hydroxyl group and of a basic amino group on opposite sides of an aromatic moiety, although adrenergic and 5-HT agonists have quite distinct structural and pharmacological properties, 5-HT_{1A}R agonists belong to diverse chemical classes but have in common a basic amino group and an aromatic ring which usually carries a hydroxyl, methoxy, or carbamoyl group which has the potential for hydrogen bond formation [7,8]. Therefore, our choices of mutants for this initial study were guided by previous studies of the human and hamster β-AR, which implicate aspartate and serine residues within several of transmembrane regions (TMR) in ligand binding [9–12]. Several recent modeling studies of this class of receptors also suggest important roles for these amino acid residues [13,14]. Based on these considerations, four mutations of aspartate, serine, or threonine residues were made: D82N (2nd TMR), D116N (3rd TMR), S198A (5th TMR), and T199A (5th TMR).

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2. MATERIALS AND METHODS

2.1. *Cell culture*

COS-7 cells were grown in a humidified environment of 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, penicillin G (100 U/ml) and streptomycin (100 μg/ml).

2.2. *Expression of the 5-HT_{1A}R*

Construction of the plasmid pTM1-5HT1AR [4], containing the 5-HT_{1A}R cDNA was described previously [4,15]. The receptor was expressed in COS-7 cells by the infection–transfection protocol [3]: 6×10^6 cells were first infected with the helper virus, vTF7-3 (multiplicity of infection of 5) which expresses the bacteriophage T7 RNA polymerase, and transfected using Lipofectin (Bethesda Research Laboratories) with 10 μg of recombinant plasmids containing the wild-type or mutant 5-HT_{1A}R cDNA driven by a T7 promoter in the specialized VV vector pTM1.

2.3. *Preparation of membranes: receptor binding and GTPase assays*

Membranes were prepared and protein levels measured as previously described [3,16]. Saturation and competitive binding studies were performed with [^{3}H]serotonin (23.2–29.8 Ci/mmol) and analyzed with the LIGAND program as described [17,18]. GTPase activity was determined by the modified method of Cassel and Selinger [19] as described [3].

2.4. *Mutagenesis*

pTM1-5HT1AR carries the f1(+) origin of replication. Single-stranded DNA of pTM1-5HT1AR was generated with the helper phage R408 (Promega) in *E. coli* CJ236. Oligonucleotide-directed mutagenesis was performed using the Bio-Rad Muta-gene M13 in vitro mutagenesis kit. The mutants were analyzed with restriction enzymes and the region around the mutation was sequenced.

2.5. *Materials*

All cell culture media were purchased from Irvine Scientific. Drugs were obtained from the following sources: serotonin HCl, [^{3}H]serotonin (Du-Pont New England Nuclear) and [γ-^{32}P]GTP (Amersham Corp. or Du Pont-New England Nuclear). All other chemicals were ultrapure or reagent grade from commercial sources.

3. RESULTS AND DISCUSSION

3.1. *Binding of normal and mutated 5-HT$_{1A}$R*

The 5-HT$_{1A}$R was previously expressed in several mammalian cell types using both the co-infection and infection–transfection variants of the VV vector/T7 RNA polymerase system [3], with co-infection giving about three-fold higher levels of expression than infection–transfection in COS-7 cells. For both methods, the affinities of the expressed 5-HT$_{1A}$R for a number of competitive inhibitors were all consistent with values observed for the endogenous 5-HT$_{1A}$R, and confirmed a 5-HT1A-type pharmacology. For studies of mutant receptors, the infection–transfection protocol is the more appropriate technique, because mutations need only be made in the plasmid pTM1–5HT1AR and need not be transferred by homologous recombination into VV.

Receptor binding assays showed that the D82N (2nd TMR), D116N (3rd TMR), and S198A (5th TMR) mutations produced a 60–100-fold decrease in affinity for 5-HT (Table I). However, affinities of the wild-type and the mutant receptors for pindolol were all approximately the same, varying by less than 2-fold (Table I, Fig. 1). Mutant T199A (also in the 5th TMR) showed insufficient specific [^{3}H]5-HT binding to yield a reliable estimate of 5-HT affinity, even though immunoblot assays using a 5-HT$_{1A}$R-specific antibody showed expression of all mutant receptors in the membrane fraction [3] (data not shown).

3.2. *GTPase activity of membrane fractions*

Because there was some variability in both the basal and maximal activity measurements in different infection–transfection experiments, GTPase activity was expressed as a percentage of the basal activity in the absence of agonist. The data in Fig. 2 show a concentration-dependent increase in activity. The EC_{50} value estimated for the wild-type receptor for 5-HT is 4.8 nM, which is close to the K_d value of 1.8 nM found in the binding assay. The mutant receptors all required a 30–100-fold higher concentration of 5-HT to induce comparable GTPase activity. Because of the limited experimental accuracy we have not attempted to estimate the

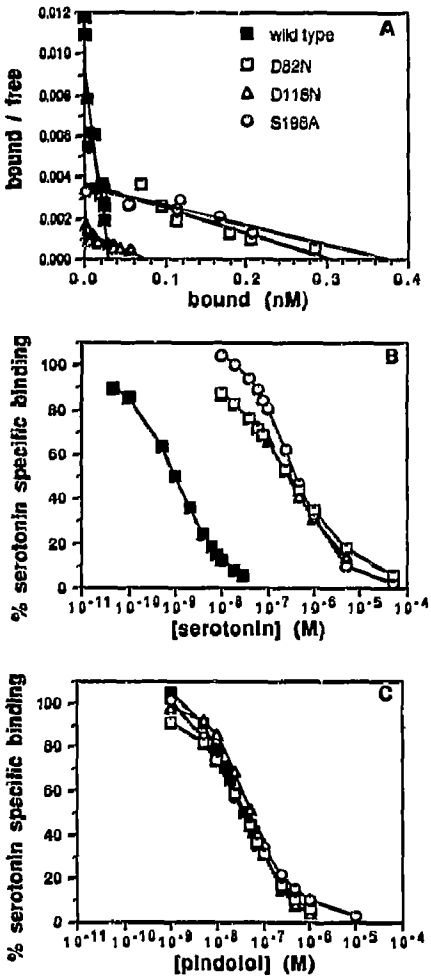

Fig. 1. Binding of serotonin (A,B) and pindolol (C) to the wild-type (WT) and mutant 5-HT$_{1A}$Rs. Saturation binding of [^{3}H]serotonin (A, Scatchard plot) and competitive binding assays of serotonin (B) or pindolol (C) were performed as described in Table I. Each curve represents the results from one experiment. The experiments were repeated at least three times with similar results.

EC_{50} values of these mutants. It is noteworthy, however, that the T199A mutation for which we were unable to detect binding by direct measurement did stimulate GTPase activity at concentrations that were only slightly higher than those observed for the other mutants. Thus, we conclude that the T199A receptor does bind agonist, but somewhat more weakly than the other mutants. The results clearly support the conclusion that all 4 mutant receptors are functional.

3.3. *Structural interpretations*

The data in the present study are in general agreement with the observations in previous studies on catecholamine receptors that the conserved aspartates in the 2nd and 3rd TMR play an important role in agonist binding [9–12,20], presumably by interaction with the protonated amino group of the agonist. These previous studies do not permit a simple interpretation as to the

relative roles of the aspartate residues in the 2nd and 3rd TMR; for the 5-HT_{1A}R studied here, the two residues seem to play approximately equal roles in binding. It was also suggested previously that the *meta*- and *para*-hydroxyl groups of the catecholamine agonists interact with S204 and S207, respectively, in the 5th TMR of the β-AR [11]. Mutations of the several serines in the 5th TMR of the D_2 receptor also decreased the binding affinity of dopamine, but S193A had the largest and S194A the smallest effect (corresponding to S198A and T199A of the 5-HT_{1A}R) [20]. We also find a significant reduction of agonist binding by the S198A mutation and by the T199A mutation. Simulation of interaction of dopamine with the D_2 receptor suggests that the agonist fluctuates rapidly between different anti and gauche conformations [13], so it is possible that the amino and hydroxyl functional groups interact with more than one residue.

Our results which indicate no effects of the four mutations on antagonist binding support the interpretation of Strader et al. [11] 'that the binding sites for agonists and antagonists are overlapping but not identical'. It has also been reported that the mutation, N385V in the 7th TMR of the 5-HT_{1A}R, decreases pindolol affinity by ca. 100-fold without affecting affinities of the agonists, 5-HT and 8-OH-DPAT [21]. These facts lead us to propose that the agonist binds in a pocket formed by the 7 transmembrane helices, with the protonated amino group of 5-HT interacting with the carboxylate groups of the aspartate residues in the 2nd and 3rd TMRs, and with the hydroxyl group on the indole ring of 5-HT interacting by hydrogen bonding with the hydroxyl groups of Ser^{198} and Thr^{199} in the 5th TMR. The data suggest different interactions with the structurally different antagonist, pindolol. It may be that an antagonist can enter the binding pocket in any one of several non-productive conformations and does not need to be positioned by simultaneous interactions with residues in the 2nd, 3rd, and 5th TMRs to compete with agonist. In any case, we find it attractive to suggest that the specific interactions of 5-HT with Asp^{82}, Asp^{116}, Ser^{198}, and Thr^{199} contribute to a conformational change in the receptor leading to signal transduction.

Table I

Affinity and expression level of wild-type and mutant 5-HT_{1A}R

	B_{max} (pmol/mg protein)	K_d (nM) serotonin	K_i (nM)	
			Serotonin	Pindolol
WT	0.82 ± 0.04	1.8 ± 0.3	1.1 ± 0.4	18 ± 2
D82N	4.2 ± 2.1	110 ± 35	240 ± 32	21 ± 6
D116N	2.4 ± 1.8	158 ± 31	198 ± 42	30 ± 5
S198A	6.9 ± 1.9	172 ± 44	250 ± 61	32 ± 6

Membranes were prepared from COS-7 cells 24 h after infection with vTF7-3 and transfection with the wild-type (WT) or mutant plasmid. B_{max} and K_d values were obtained from saturation binding using [^{3}H]serotonin. Competitive binding assays were performed with 1 nM [^{3}H]serotonin for the wild-type receptor and 50 nM [^{3}H]serotonin for the mutants. K_i values for serotonin and pindolol have been corrected for the serotonin K_d values obtained from the saturation binding assays. Non-specific binding was determined in the presence of 10 μM serotonin for the wild-type and 50 μM serotonin for the mutants. Data are expressed as mean ± S.E.M. from at least three independent experiments.

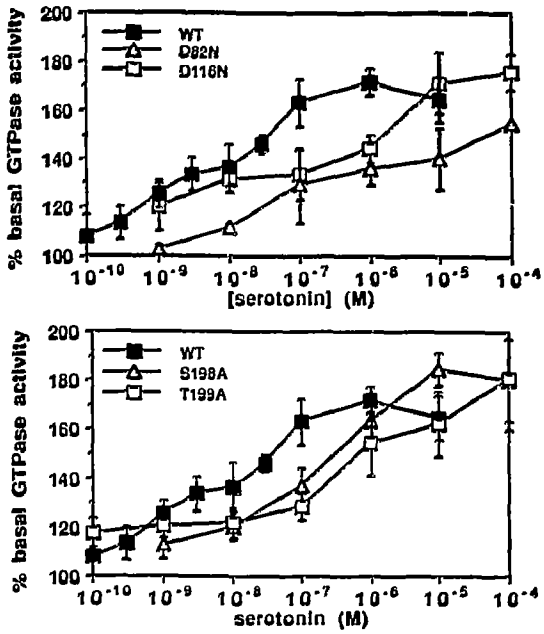

Fig. 2. Stimulation of GTPase activity by serotonin for wild-type (WT) and mutant 5-HT_{1A}Rs. Membranes were prepared from COS-7 cells 24 h after infection with vTF7-3 and transfection with the wild-type or mutant plasmid. Average basal low K_m GTPase activities were 13.9 ± 3.8 (WT), 23.0 ± 4.5 (D82N), 29.13 ± 13.3 (D116N), 14.5 ± 5.16 (S198A), 14.7 ± 1.5 (S199A) pmol/mg protein/min. The same wild-type results are shown in both figures. Data of low K_m GTPase activity levels were expressed as a percentage of their respective basal value from each assay. Data shown are mean ± S.E.M. from at least three separate infection–transfection experiments.

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