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Conserved His^{VI-17} of the NK-1 receptor is involved in binding of non-peptide antagonists but not substance P

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Residue number 17 in transmembrane segment VI has been shown to be crucial for the binding of agonists in G-protein-coupled receptors for the monoamines. In many peptide receptors a histidyl residue has been conserved at this position. We find that replacement of His^{VI-17} in the NK-1 receptor with either glutamine, phenylalanine, or alanine has no apparent effect on the binding of the natural peptide ligand substance P or on the agonist induced increase in inositolphosphate turnover. However, the binding of certain non-peptide antagonists was impaired; for example, replacement of His^{VI-17} with alanine decreased the affinity for FK888 and RP67,580 5- to 12-fold, respectively. A glutamine side chain was a good substitute for the imidazole in the binding of all non-peptide antagonists. It is concluded that the conserved His^{VI-17} in the NK-1 receptor is involved in the binding of certain non-peptide antagonists, but is not important for the action of the natural peptide agonist, substance P.

G-protein-coupled receptor; Tachykinin; Substance P; Non-peptide antagonist; Mutagenesis

1. INTRODUCTION

In the large superfamily of G-protein-coupled receptors, ligand binding sites for monoamine transmitters have been characterized in rather great detail. Through a combination of molecular biology and medicinal chemistry an ionic interaction between the amine function of the monoamines and the Aspartate residue located in position 8 in transmembrane segment III (Asp^{III-8} in TM-III) has been shown to be crucial for high affinity binding of the ligand [1]. The activation of the receptor is apparently obtained through the interaction of the catechol ring with residues in TM-V and -VI. The catechol ring appears to be correctly orientated through hydrogen-bonding with two serine residues in TM-V, Ser^{V-4} and Ser^{V-7}, thus providing the appropriate interaction between the aromatic ring and the hydrophobic residues in TM-VI; in the case of the β-adrenergic receptor, this would especially involve Phe^{VI-17} [2,3]. Molecular models have been developed in which residues located at the corresponding positions in other receptors are imagined to be involved in the binding of many other classes of ligands [4,5]. In the case of receptors for serotonin (5HT), it has recently been demonstrated by mutagenesis that Phe^{VI-17} is important for the binding of the agonists, and of certain antagonists which are structurally similar to the agonists [6]. Based on molecular modeling it has been speculated that the corresponding residue, His^{VI-17}, in the adenosine receptors, could be involved in the binding of the carbohydrate moiety of the adenosine agonist [7]. As shown in Fig. 1, in most peptide receptors residue number 17 in TM-VI is either a histidyl residue or another polar residue, that might similarly be involved in the binding of the polar peptide ligands.

Substance P is an important neuropeptide, which is believed to play an important role in afferent transmission of sensory stimuli and for the neurogenic contribution to especially cronic inflammatory processes [8–10]. A series of non-peptide antagonists have been developed, which specifically and with high affinity block the substance P receptor, NK-1 [10]. These compounds, which do not structurally resemble substance P, have been discovered mainly through screening of chemical files. Recently, the binding sites for a series of non-peptide NK-1 antagonists have been located to a discontinuous epitope around the outer portion of TM-V and -VI [11,12] (and Gether et al. submitted for publication). Here, we study the importance of His^{VI-17}, which has been suggested to be part of the binding site for substance P [13], by replacing it with, respectively, alanine, glutamine or phenylalanine. Surprisingly, this histidyl residue appears to be of no importance for agonist binding or receptor activation, while it is clearly involved in the binding of the non-peptide antagonists.

2. MATERIALS AND METHODS

2.1. *Peptide and non-peptide ligands*

Substance P was purchased from Peninsula (St. Helens, Merseyside, UK). The following non-peptide compounds were used: CP 96,345, (2*S*,3*S*)-*cis*-2-diphenylmethyl)-*N*[(2 -methoxyphenyl)methyl]-1-azabicyclo[2.2.2]octan-3-amine supplied by John A. Lowe III, Pfizer, Groton, CT [14]; SR 140,133, (S) 1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl}-4-phenyl-1-azabicyclo-[2.2.2]octan, chloride supplied by Xavier Emonds-Alt, Sanofi Recherche, F-34184 Montpellier, France [15]; FK888, N^2-[(4*R*)-4-hydroxy-1-[(1-methyl-1*H*-indole-3-yl)-L-propyl]-*N*-methyl-*N*-(phenyl-

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methyl)-3-(2-naphthyl)-L-alaninamide supplied by Takashi Fujii, Fujisawa Pharmaceutical Co., Osaka, Japan [16]; and RP67,580, 2-[1-imino-2-(2-methoxyphenyl)-ethyl]-7,7-diphenyl-4-perhydroisoindolone (3aR,7aR) supplied by Laurant Pradier, Rhône-Poulenc Rorer, Vitry sur Seine, France [17].

2.2. *Site-directed mutagenesis*

Substitution of His^{VI-17}, corresponding to His^{265}, in the human NK-1 receptor (cDNA generously supplied by Drs. Norma and Craig Gerard) with Ala, Gln, or Phe was performed by extension of mutated internal primers using T4 DNA polymerase followed by selective amplification of the mutated DNA by the polymerase chain reaction (PCR) according to methods described [18]. Briefly, an initial synthesis reaction was carried out with T4 polymerase and T4 DNA ligase after the annealing of both a sense oligonucleotide containing the desired mutation and an upstream oligonucleotide with a non-homologous 5′ add-on sequence to a single-stranded M13 template of the human NK-1 receptor cDNA. The mutated DNA strand was thereafter selectively amplified by PfuPolymerase-mediated PCR using a sense primer corresponding to the non-homologous 5′ add-on sequence of the upstream primer and a non-sense primer located downstream of the mutation site. The cDNA encoding the wild-type or the mutated receptors were cloned into the pTEJ-8 expression vector [19]. The structure of the recombinant genes was verified by restriction endonuclease mapping and by DNA sequence analysis.

2.3. *Transfections and tissue culture*

The expression plasmids containing the cDNAs encoding the wild-type NK-1 and the mutant receptors were transiently transfected into COS-7 cells by the calcium phosphate precipitation method according to previously reported methods [11,19].

2.4. *Binding experiments*

Monoiodinated ^{125}I-Bolton Hunter-labeled substance P ([^{125}I]BH-SP) was prepared and purified by HPLC as described in detail previously [20]. The transfected COS-7 cells were transferred to 12-well culture plates, $0.1–0.6 \times 10^5$ cells/well, 1 day after transfection and 24 h before performing the binding experiments, as described. The number of cells per well was adjusted according to the expression efficiency of the individual plasmid aiming at 5–10% binding of the added radioligand in the competition binding experiments. Binding experiments were performed for 3 h at 4°C with 50 pM [^{125}I]BH-SP plus variable amounts of unlabeled peptide or non-peptide compound present in 0.5 ml of a 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 5 mM $MnCl_2$, and 0.1% (w/v) bovine serum albumin (Sigma) supplemented with 100 μg/ml bacitracin (Sigma). All determinations were performed in triplicate and the non-specific binding was determined as the binding in the presence of 1 μM substance P. The specific binding constituted more than 80% of the total binding. The binding data were analyzed and IC_{50} values determined by computerized non-linear regression analysis using InPlot (GraphPad Software, San Diego, CA). K_D, B_{max}, and K_I values for binding of radiolabeled substance P to the different receptors were estimated from competition binding experiments using different concentrations of the unlabeled peptide as indicated in the figures [20].

2.5. *Inositol phosphate turnover*

COS cells were seeded in 12-well culture plates at a density of 5×10^5 cells/well and incubated with [^{3}H]*myo*-inositol (2.5 μCi/well) (PT6-271 from Amersham, Little Chalfont, UK) for 24 h in inositol-free Dulbecco's 1885 medium supplemented with 10% fetal calf serum, 2 mM glutamine and 0.1 mg/ml gentamicin. The cells were washed twice in assay buffer (20 mM HEPES, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1mM $CaCl_2$, 10 mM glucose, and 0.05% BSA) and preincubated for 30 min at 37°C in 0.5 ml assay buffer containing 10 mM LiCl before addition of peptide agonists. The antagonists were added 5 min prior to the addition of peptide agonist. After 20 min of incubation at 37°C the cells were extracted by addition of 0.5 ml ice-cold 10% perchloric acid followed by incubation on ice for 30 min. The resulting supernatants were neutralized and the generated [^{3}H]inositol phosphates were separated from [^{3}H]inositol using of a slurry of Bio-Rad AG 1-X8 anion-exchange resins, 100–200 mesh, formate form (Bio-Rad Laboratories, Hercules, CA) according to principles previously described [21].

3. RESULTS

The binding of substance P was unaffected by the substitution of His^{VI-17} in the human NK-1 receptor with either Ala, Gln, or Phe, as reflected in unaltered competition binding experiments using radiolabeled substance P (Fig. 2). Furthermore, substance P stimu-

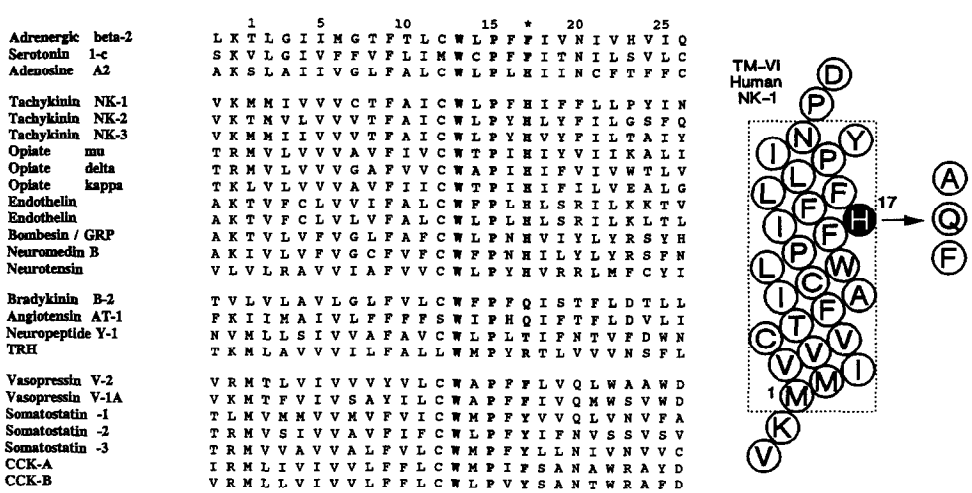

Fig. 1. Structure of transmembrane segment VI (TM-VI) of the human NK-1 receptor. (Right panel) The position of His^{VI-17} (His^{265}) in the presumably helical structure of TM-VI of the human NK-1 receptor. (Left panel) Alignment of the amino acid sequences of a representative adrenergic, serotinergic and an adenosine receptor plus a series of peptide receptors. The numbering of the residues in the helix is according to the nomenclature of Hibert et al. [4,5]. In all adrenergic and serotinergic receptors residue, VI-17, is a Phe; in all three adenosine receptors it is a His; and in most neuropeptide receptors of the rhodopsin family it is either a His residue or another polar residue [26].

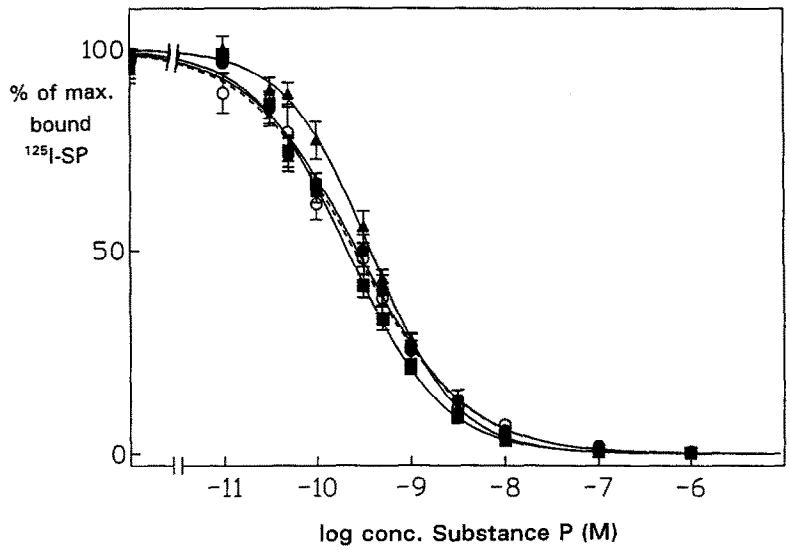

Fig. 2. Substance P (SP) binding in His^{VI-17} substituted human NK-1 receptors. Competition binding experiments using ^{125}I-labeled substance P in wild-type hNK-1 receptor (– – –), [Gln^{VI-17}]hNK-1 (●), [Ala^{VI-17}]hNK-1 (■), or [Phe^{VI-17}]hNK-1 (▲) expressed transiently in COS-7 cells.

lated inositolphosphate turnover to a similar degree in COS-7 cells transiently expressing the wild-type NK-1 receptor or any of the mutant NK-1 receptors with substitutions in position VI-17 (Fig. 3).

The binding of the non-peptide antagonist, CP96,345, has previously been shown not to be affected by chimeric exchanges of non-conserved residues around the top of TM-VI in the rat NK-1 receptor [11]. However, when the conserved His^{VI-17} at the top of TM-VI was replaced by Ala in the human receptor, an increase in the K_I value for CP96,345 from 0.99 to 2.8 nM was observed (Fig. 4, lower left panel; Table I). The binding of SR140.333 was not affected by this substitution (Fig. 4, lower right panel). However, in the case of the two other non-peptide antagonists, FK888 and RP67,580, the His-to-Ala substitution increased their K_I values by a factor of 5 and 12, respectively (Fig. 4, upper two panels; Table I). Gln was a good substitute for His at position VI-17 in the NK-1 receptor, with no loss of affinity for any of the non-peptide antagonists (Fig. 4; Table I), whereas replacement of the His residue with Phe yielded intermediate results between those observed with the Ala- and the Gln-substituted receptors (Table I). In all cases, the non-peptide compounds still functioned as antagonists of the substance P-induced increase in inositolphosphate turnover, although the response to the antagonists appeared to be slightly less in the Gln^{VI-17}-substituted receptor (Fig. 3).

4. DISCUSSION

His^{VI-17} in the NK-1 receptor corresponds to Phe^{VI-17}

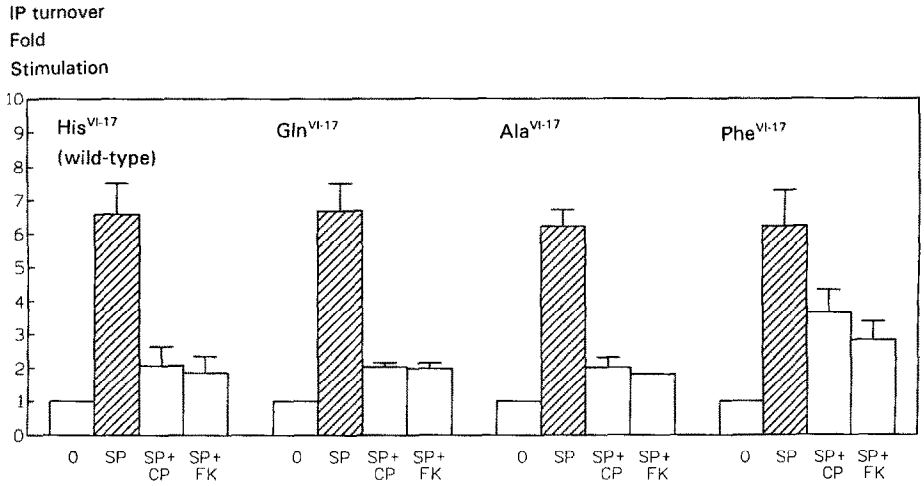

Fig. 3. Stimulation and inhibition of inositol phosphate turnover in COS cells expressing wild-type and His^{VI-17} substituted human NK-1 receptors. Inositol phosphate turnover was determined as described in the text in COS cells transiently expressing wild-type hNK-1 receptor ($n = 3$), [Ala^{VI-17}]hNK-1 ($n = 2$), [Gln^{VI-17}]hNK-1 ($n = 2$), or [Phe^{VI-17}]hNK-1 ($n = 3$). Cells were stimulated with a submaximal dose of substance P (10^{-8} M) alone (SP) or together with 10^{-6} M CP96,345 (SP + CP) or 10^{-6} M FK888 (SP + FK). Means ± S.E.M. are shown.

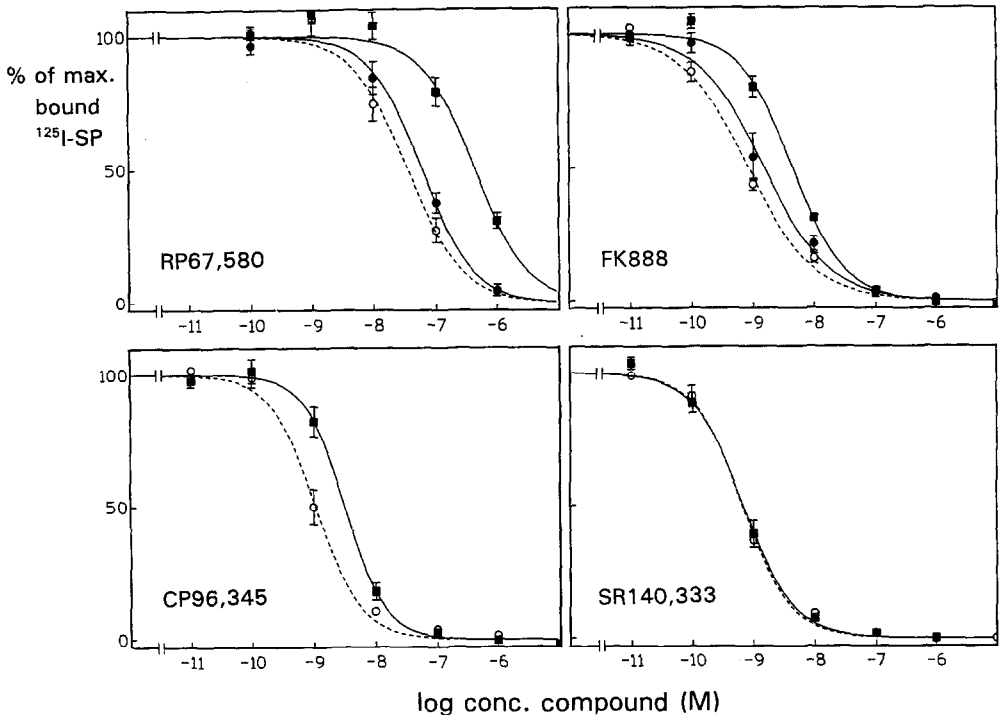

Fig. 4. Non-peptide antagonist binding in His^{VI-17} substituted human NK-1 receptors. Competition binding experiments with radiolabeled substance P are shown for RP 67,580 (upper left), FK 888 (upper right), CP 96,345 (lower left) and SR 140,333 (lower right), in the wild-type human NK-1 receptor (– – –) and in the [Ala^{VI-17}]hNK-1 (■) and in the [Gln^{VI-17}]hNK-1 (●).

of the monoamine receptors, which has previously been shown by mutagenesis to be involved in agonist binding [3,6]. A His residue or another polar residue has been conserved at this position in most peptide receptors of the G-protein-coupled class. This imidazol side chain could be envisioned to function as a hydrogen bond donor and/or acceptor in the binding of the polar peptides like substance P [13]. Nevertheless, in the present study we find that this His residue apparently is not involved in the binding of the natural peptide ligand, substance P. Recently we have made a similar observation in the angiotensin AT_1 receptor, where a His residue at position 16 of TM-VI (see Fig. 1) can be replaced by Gln, Asn, Phe, or Ala, without loss of binding affinity for angiotensin (Schambye, Hjorth and Schwartz, to be published). Since residues in this area are important for the binding of several small-sized chemical messengers [3,6], our observations could indicate that peptides may be able to activate their receptors without reaching – at least at this particular point – deep in between the transmembrane segments. In fact, all mutagenesis studies in peptide receptors indicate that important interaction points for peptide agonists are located in the superficial parts of the receptors. Thus, it has been known for a couple of years that the large glycoprotein hormones mainly bind to the large N-terminal segment of their target receptors [23,24]. Key points of interaction for the relatively small neuropeptides and peptide hormones, like substance P, bombesin, and angiotensin, have only been located in the extracellular loops, in the outermost parts of the transmembrane segments, as well as in the N-terminal extracellular extention of their receptors [22,25] (and Hjorth, Schambye and Schwartz, to be published).

Table I

K_I values (nM) for four non-peptide antagonists determined by competition binding experiments with ^{125}I- labeled substance P in COS cells transiently expressing the human NK-1 receptor or three mutant forms of this with single substitutions of His^{VI-17} (His^{265})

	Substance P (K_D)	RP67,580	FK888	CP96,345	SR140,333
hNK-1	0.29 ± 0.05	33 ± 3	0.72 ± 0.07	0.92 ± 0.18	0.60 ± 0.07
[Gln^{VI-17}]hNK-1	0.28 ± 0.05	58 ± 7	1.72 ± 0.66	1.53 ± 0.35	0.47 ± 0.06
[Ala^{VI-17}]hNK-1	0.18 ± 0.03	360 ± 70	4.2 ± 0.2	2.8 ± 0.6	0.59 ± 0.13
[Phe^{VI-17}]hNK-1	0.36 ± 0.03	95 ± 7	2.2 ± 0.5	1.33 ± 0.35	0.58 ± 0.07

The K_D values for substance P as determined by competition binding experiments are also listed.

In contrast to the peptide agonist, the binding of several non-peptide antagonists was impaired by the substitution of His^{VI-17}. This decrease in binding affinity was similar in magnitude to that recently described for CP96,345 upon substitution of a His residues located at the top of TM-V [12]. In the present study, we have not made any attempt to identify which part of the RP67,580 and FK888 compounds interact with His^{VI-17} of the NK-1 receptor. However, this problem can be addressed by combining a systematic series of compound analogs with a library of mutant receptors. Recently, Fong and co-workers in this way found evidence for what they interpreted as an amino–aromatic interaction between the biphenyl group of CP96,345 and the other His residue located at the top of TM-V of the NK-1 receptor (His^{197} or His^{V-0}) [12]. In that case, a glutamine residue was a good substitute with respect to the interaction with the non-peptide antagonists, just like it was in the present study for His^{VI-17}.

Since His^{VI-17} does not appear to be involved in the binding of the natural ligand, the question still remains as to why a His residue or a 'similar' polar residue has been conserved at this particular position in the majority of peptide receptors of the G-protein-coupled class. Possibly, it is simply favorable to have polar residues facing inward, i.e. away from the lipids, for stabilization of the receptor structure whenever an aromatic residue is not needed at this position for agonist binding.

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