British Journal of Pharmacology (2000) 131, 1247–1250
www.nature.com/bjp

SPECIAL REPORT

Distinct pharmacology of rat and human histamine H_3 receptors: role of two amino acids in the third transmembrane domain

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Starting from the sequence of the human histamine H_3 receptor (hH_3R) cDNA, we have cloned the corresponding rat cDNA. Whereas the two deduced proteins show 93.5% overall homology and differ only by five amino acid residues at the level of the transmembrane domains (TMs), some ligands displayed distinct affinities. Thioperamide and ciproxifan were about 10 fold more potent at the rat than at the human receptor, whereas FUB 349 displayed a reverse preference. Histamine, (R)α-methylhistamine, proxyfan or clobenpropit were nearly equipotent at H_3 receptors of both species. The inverse discrimination patterns of ciproxifan and FUB 349 were partially changed by mutation of one amino acid (V122A), and fully abolished by mutation of two amino acids (A119T and V122A), in TM3 of the rH_3R located in the vicinity of Asp^{114} purported to salt-link the ammonium group of histamine. Therefore, these two residues appear to be responsible for the distinct pharmacology of the H_3R in the two species.
British Journal of Pharmacology (2000) **131,** 1247–1250

Keywords: Histamine; H_3 receptor; G protein-coupled receptors; ciproxifan; thioperamide; [^{125}I]iodoproxyfan; FUB 349; clobenpropit; site-directed mutagenesis

Abbreviations: H_3R, histamine H_3 receptor; hH_3R, human histamine H_3 receptor; rH_3R rat histamine H_3 receptor; TM, transmembrane domain

Introduction Whereas the histamine H_3 receptor (H_3R) was initially identified in the rat brain (Arrang *et al*., 1983; 1987), its presence in the human brain was confirmed a few years later (Arrang *et al*., 1988). In both cases a functional test, the inhibition of [^{3}H]-histamine release from depolarized brain slices, was used, but the pharmacological characterization of the human H_3R has remained preliminary since the availability of fresh brain tissues obtained during neurosurgery is limited. Nevertheless there were some indications that the pharmacology of the human and the rat H_3R may slightly differ (Arrang *et al*., 1988; West *et al*., 1999 and X. Ligneau, unpublished observation).

With the recent cloning of the human H_3R (hH_3R) (Lovenberg *et al*., 1999), it became feasible to determine with greater precision the apparent affinity of ligands at this receptor and assess the existence of species differences. Namely, for this purpose we have cloned the rat H_3R (rH_3R) starting from the published sequence of the hH_3R and established permanent cell lines expressing the hH_3R or rH_3R. This allowed us to identify ligands displaying distinct apparent affinities at the H_3R of the two species. Then we tried to identify the amino acid residues responsible for such discrimination using site-directed mutagenesis of the rH_3R.

Methods *Cloning of the rH_3R cDNA* A rat striatal cDNA library (4×10^6 phages; Stratagene, St-Quentin-en-Yvelines, France) was screened at high stringency with a ^{32}P-labelled fragment (607 bp), obtained by RT–PCR amplification of total mRNAs from rat cerebral cortex using primers based on the sequence of the third transmembrane domain (TM3 nucleotides 299–331) and the third intracellular loop (nucleotides 1601–1637) of the hH_3R, respectively (Lovenberg *et al*., 1999). Bluescript KS(+) plasmids were recovered from sixty positive clones and their cDNA inserts sequenced. Some of them exhibited a full-length open reading frame encoding a 445-amino acid protein corresponding to the rH_3R.

Cloning of the hH_3R cDNA Screening of a human striatum cDNA library (Stratagene) with the same probe led to the isolation of five positive clones. Among them, one exhibited a full-length cDNA sequence displaying a 100% identity with the hH_3R cDNA recently described (Lovenberg *et al*., 1999).

Stable transfection of CHO-K1 cells cDNA inserts corresponding to the full-length coding sequences of the rH_3R and hH_3R, were ligated into the mammalian expression vector pCIneo (Promega, Charbonnières, France). CHO-K1 cells were transfected using SuperFect (Qiagen, Courtaboeuf, France). Stable transfectants were selected with 2 mg ml^{-1} of G418 and tested for [^{125}I]-iodoproxyfan binding (Ligneau *et al*., 1994). Two clones expressing ~300 fmol mg^{-1} protein of rH_3R and hH_3R binding sites were selected for further characterization and maintained in the presence of 1 mg ml^{-1} of G418.

Site directed mutagenesis of the rH_3R Rat H_3-receptor mutants were constructed using the Transformer Site-Directed Mutagenesis kit (Clontech, St-Quentin-en-Yvelines, France). The rH_3R cDNA subcloned into pCIneo plasmid (0.2 μg) was used for synthesis of mutant cDNA strands using T4 DNA polymerase (4 units; Clontech) and T4 DNA ligase (6 units; Clontech), in the presence of the mutagenic and selection primers phosphorylated with T4 polynucleotide kinase (Roche,

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Meylan, France). The mutagenic primer (5′-cctactgtgtacctcctcggccttcaacatc-3′) was designed to introduce the required mutations within the third transmembrane domain of the rH_3R and to suppress a *Bbs*I restriction site which was found in the wild-type rH_3R sequence. The selection primer (5′-cgagacagaaaaaacacttgcgtttctgata-3′) was designed to suppress a *Bbs*I restriction site found within the pCIneo plasmid sequence. Following selection by *Bbs*I restriction endonuclease digestion, the mutant plasmids were amplified in competent BMH71-18*mutS* cells (Clontech) and further isolated after complete *Bbs*I digestion of parental plasmids (40 units; 2 h). Following amplification in Top10 cells (Invitrogen, Groningen, The Netherlands), undigested (mutant) plasmids were prepared and sequenced using Taq FS DNA polymerase (Perkin-Elmer, Courtaboeuf, France).

Transient expression in Cos-1 cells Cos-1 cells were grown to 40% confluence in DMEM-Ham F12 medium (Life Technologies, Cergy-Pontoise, France) with 10% foetal calf serum (Valbiotech, Paris, France) in 96 mm Petri dishes and transfected with 5 μg of the plasmid pCIneo-rH_3R, pCIneo-[122A]rH_3R, pCIneo-[119T/122A]rH_3R and pCIneo-hH_3R, using 30 μl of SuperFect Transfection Reagent (Qiagen). Two days later, the cells were harvested and membranes prepared for binding assays.

[^{125}I]-Iodoproxyfan binding assays Transfected CHO or Cos-1 cells were washed and homogenized with a polytron in ice-cold binding buffer (Na_2HPO_4/KH_2PO_4 50 mM, pH 6.8) and assays performed as described (Ligneau *et al*., 1994). Briefly, aliquots of membrane suspension (5–15 μg of protein) were incubated for 60 min at 25°C with 25 pM [^{125}I]-iodoproxyfan alone, or together with competing drugs (200 μl, final volume). The nonspecific binding was determined using imetit (1μM).

Analysis of data IC_{50} values were determined using an iterative least-squares method derived from that of Parker & Waud (1971). K_i values of ligands were calculated from their IC_{50} values, assuming a competitive antagonism and by using the relationship (Cheng & Prussoff, 1973):

$$K_i = IC_{50}/(1 + (S/K_D)) \text{ where}$$

S and K_D represent the concentration and dissociation constant of the radioligand respectively.

Results *Comparison of the rH_3R and hH_3R amino acid sequences* Sequence analysis of the rH_3R cDNA revealed a full-length open reading frame encoding a protein of 445 amino acids displaying 93.5% overall homology with the human receptor. Amino acid sequence alignments of the two cloned receptors showed only five different residues within the seven transmembrane domains (TMs). Among the latter, two were found in TM3, close to an aspartic acid residue known to be conserved in all aminergic receptors, and corresponded to A119T and V122A transitions (Figure 1).

Comparison of the rH_3R and hH_3R pharmacological profiles in CHO cells Specific [^{125}I]-iodoproxyfan binding to membranes of CHO(rH_3R) and CHO(hH_3R) was monophasic and saturable (B_{max} ~300 fmol mg^{-1} protein). Computer analysis by nonlinear regression using a one-site cooperative model led to K_D values of 68±15 pM and 50±7 pM at rH_3R and hH_3R, respectively. [^{125}I]-Iodoproxyfan binding was inhibited by a series of H_3-receptor ligands with K_i values defining a pharmacological profile of the rH_3R distinct from that of the hH_3R (Table 1). Histamine and the agonist (R)α-methylhistamine displaced specific binding ($n_H = 0.7-0.8$) with similar affinities at the two receptors. The pseudo Hill coefficient of the antagonists did not significantly differ from unity. Among the latter, proxyfan and clobenpropit displayed a similar affinity at the two receptors, whereas thioperamide and ciproxifan were about 10-fold more potent at the rH_3R and, in contrast, FUB 349 was about 6 fold more potent at the hH_3R (Table 1).

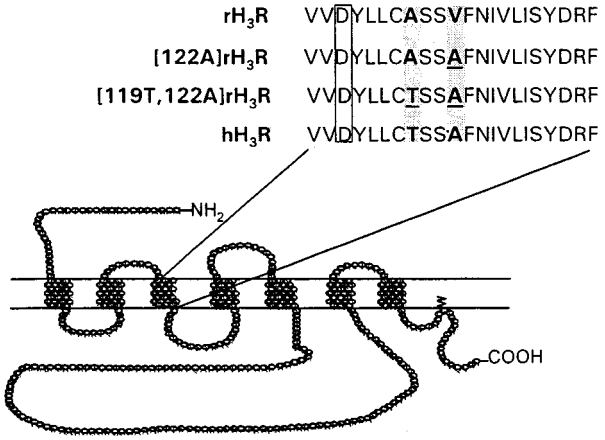

Figure 1 Putative membrane topology of the histamine H_3 receptor. The amino acid sequence of the third transmembrane domain of the rat (rH_3R) and the human (hH_3R) receptor is shown. The open box indicates the position of aspartic acid 114, known to be conserved in all aminergic receptors. The grey boxes indicate the amino acids in position 119 and 122. Mutations at these position in the rat receptor are underlined.

Pharmacological analysis of mutant rat receptors in Cos-1 cells Sequence analysis of mutant plasmids obtained by site-directed mutagenesis revealed the two expected mutations of the rat receptor, i.e., mutation of alanine 119 to threonine and mutation of valine 122 to alanine ([119T, 122A] rH_3R). However, one set of plasmids contained only the mutation of valine 122 ([122A] rH_3R) (Figure 1). [^{125}I]-Iodoproxyfan binding to membranes of Cos-1 cells expressing rH_3R, [122A] rH_3R, [119T, 122A] rH_3R and hH_3R occurred with similar K_D values, i.e. 71±4; 57±13; 45±5 and 41±6 pM, respectively. As with CHO cells, ciproxifan inhibited H_3R binding to Cos-1 cell membranes with higher potency at the wild-type rat, as compared to the human receptor (13 fold difference). This difference was reduced with the single and abolished with the double mutation) (Figure 2).

The opposite difference in potency of FUB 349 in wild-type receptors detected in CHO cells was confirmed in Cos-1 cells (~5 fold higher potency at hH_3R than at rH_3R) and, this time, the mutations resulted in enhanced potency (over 10 fold in the double mutation) (Figure 2).

In contrast, the potency of clobenpropit was similar at the H_3R of the two species expressed in Cos-1 cells, i.e., 1.5–2 nM (not shown).

Discussion Using recombinant receptors expressed in different cell lines, the present work first confirms that the H_3Rs can be differentiated pharmacologically in two species and, then, identifies the area very likely responsible for this difference.

Previous indications of such species differences, mainly between the hH_3R and rH_3R were derived from either functional or binding assays performed with fresh brain tissues. In agreement, the prototypical H_3R antagonist thioperamide was found to be slightly (4 fold) less potent at the H_3R modulating [^{3}H]-histamine release from depolarized

Table 1 Compared potencies of H_3-receptor ligands on inhibition of [^{125}I]-iodoproxyfan binding to rH_3R and hH_3R stably expressed in CHO cells

Compounds	*Structure*	*K_i (nM)* rH_3R	hH_3R
Agonists			
Histamine	Im—$(CH_2)_2$—NH_2	20 ± 2	13 ± 2
(R)α-methylhistamine	Im—CH_2—$CH(CH_3)$—NH_2	3.6 ± 0.4	2.7 ± 0.9
Antagonists (equipotent)			
Proxyfan	Im—$(CH_2)_3$—O—CH_2—Ph	2.9 ± 0.2	2.7 ± 0.1
Clobenpropit	Im—$(CH_2)_3$—S—C(=NH)—NH—CH_2—Ph—Cl	1.4 ± 0.1	2.4 ± 0.6
Antagonists (discriminant)			
Thioperamide	Im—Cy—N—C(=S)—NH—Ph	6.5 ± 0.2	60 ± 12
Ciproxifan	Im—$(CH_2)_3$—O—Ph—C(=O)—Cyclopropyl	3.9 ± 0.2	46 ± 4
FUB 349	Im—$(CH_2)_4$—Ph	12 ± 1	2.1 ± 0.2

Im = Imidazole substituted in 4-position. Means ± s.e.mean of 3 independent experiments with triplicate determinations.

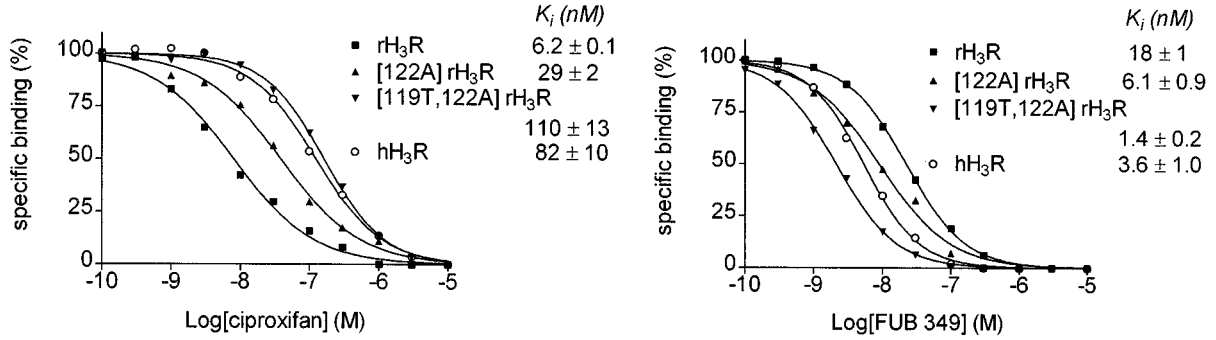

Figure 2 Inhibition of [^{125}I]-iodoproxyfan binding to mutant rat receptors by ciproxifan and FUB 349. Membranes of Cos-1 cells expressing wild-type rat receptors (rH_3R), mutant [122A] rat receptors, mutant [119T, 122A] rat receptors or wild-type human receptors (hH_3R) were incubated with 25 pM [^{125}I]-iodoproxyfan and ciproxifan or FUB 349 in increasing concentrations. Each point represents the mean value from two different experiments with triplicate determinations each. The K_i values (nM) of ciproxifan and FUB 349 obtained for each receptor are indicated.

human when compared to rat brain slices, K_i values being 16 nM (Arrang *et al.*, 1988) and 4 nM (Arrang *et al.*, 1987), respectively. Other functional or binding studies performed with various ligands led to even higher K_i values for this compound at the hH_3R, i.e., 85–200 nM (Cherifi *et al.*, 1992; West *et al.*, 1999). In the same way the antagonist ciproxifan displayed significantly higher potency at the rH_3R when compared to the hH_3R in fresh brain tissues using either functional or binding assays performed under parallel conditions (Ligneau *et al.*, 1998 and X. Ligneau, unpublished observations). Interestingly, however, both histamine and (R)α-methylhistamine were found to be nearly equipotent at the native rH_3R and hH_3R in brain (Arrang *et al.*, 1987, 1988; West *et al.*, 1999).

Here we come to similar conclusions, i.e., thioperamide and ciproxifan are about 10 fold more potent at the rH_3R than at the hH_3R, whereas histamine, (R)α-methylhistamine and the two antagonists clobenpropit (Van der Goot *et al.*, 1992) and proxyfan (Stark *et al.*, 1998a) were nearly equipotent.

Moreover, we identified one compound, the antagonist FUB 349 (Stark *et al.*, 1998b), displaying a reverse preference, i.e., being about 5 fold more potent at the human than at the rat receptor.

While this work was in progress, Lovenberg *et al.* (2000) have also cloned a rH_3R with a sequence 100 per cent identical to that we have established independently, and confirmed the higher potency of thioperamide (K_i = 4 nM) at this receptor when compared to its human

counterpart (K_i = 58 nM or K_i = 20 nM in Lovenberg *et al*., 1999).

Although these differences in potency of some H_3R ligands in the two species are of rather limited amplitude, they are reproducibly found with native as well as recombinant receptors expressed in various cell lines. This leaves little doubt about the existence of a pharmacological heterogeneity.

The identification of residues responsible for this heterogeneity was facilitated by the realization that the rH_3R and hH_3R sequences differed by only five amino acid residues, at the level of the putative TM helices where ligands are thought to bind. Among these helices, TM3 was a good candidate since, at this level, the rH_3R differs from the hH_3R by two residues located in vicinity to the aspartate residue (Asp^{114}) present in all aminergic receptors and purported to salt-link the ammonium group of histamine and agonists. Mutation of these two residues, i.e., Ala^{119} into Thr^{119} and Val^{122} into Ala^{122}, to obtain a partially 'humanized' rat H_3 receptor led to the expected changes. In agreement: (i) the affinity of ligands not discriminating hH_3R and rH_3R, e.g., [^{125}I]-iodoproxyfan or clobenpropit was not significantly modified, and (ii) in contrast the affinity of a rH_3R-preferring ligand, ciproxifan, was reduced, whereas that of FUB 349, a hH_3R-preferring ligand, was enhanced so that the affinity of these compounds did not differ anymore from corresponding values at the hH_3R (Figure 2).

The mutated amino acids may modify, e.g., hydrophobic interactions, which are presumed to have greater influence on the binding of lipophilic antagonists than on that of hydrophilic agonists. The purported salt link of basic compounds like histamine, (R)α-methylhistamine, and clobenpropit with Asp^{114} seems to be of greater importance for these compounds than their hydrophobic interactions. Such hydrophobic interactions may involve the mutated transmembrane domain and the relative potencies at rat and human receptors be determined by the relative positioning of the imidazole ring, the phenyl (or other hydrocarbon residue) and the polar group, if present in the antagonist.

Interestingly, the guinea-pig H_3R slightly differs from both hH_3R and wild-type or mutated rH_3R by the presence of threonine and valine residues at corresponding 119 and 122 positions respectively (Tardivel-Lacombe *et al*., 2000). Since the isolated guinea-pig ileum is currently used to evaluate the potency of H_3R ligands (Hill *et al*., 1997), it would be interesting to assess the role of the two critical TM3 amino acid residues in differences in H_3R pharmacology in this species, if any.

In addition, more extensive structure-activity together with modelling studies are likely to provide more details about a possible interaction of ligands with the receptor at this level and should facilitate the rational design of novel ligands to be used as drugs in humans.

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(Received August 4, 2000
Revised September 12, 2000
Accepted September 15, 2000)