Ligand-Specific Contribution of the N Terminus and E2-Loop to Pharmacological Properties of the Histamine H₁-Receptor

Andrea Straßer, Hans-Joachim Wittmann, and Roland Seifert

ABSTRACT

There are species differences between human histamine H₁ receptor (hH₁R) and guinea pig (gp) histamine H₁ receptor (gpH₁R) for phenylhistamines and histaprodifens. Several studies showed participation of the second extracellular loop (E2-loop) in ligand binding for some G protein-coupled receptors (GPCRs). Because there are large species differences in the amino acid sequence between hH₁R and gpH₁R for the N terminus and E2-loop, we generated chimeric hH₁Rs with gp E2-loop (hgpE2H₁R) and gp N terminus and gp E2-loop (hgpNgpE2H₁R). hH₁R, gpH₁R, and chimeras were expressed in Sf9 insect cells. [³H]Mepyramine binding assays and steady-state GTPase assays were performed. In the series hH₁R > hgpE2H₁R > hgpNgpE2H₁R, we observed a significant decrease in potency of histamine 1 in the GTPase assay. For phenoprodifen 6R and the chiral phenoprodifens 6R and 6S, a significant decrease in affinity and potency was found in the series hH₁R > gpH₁R > hgpNgpE2H₁R. In addition, we constructed new active-state H₁R models based on the crystal structure of the human β₂-adrenergic receptor (hβ₂AR). Compared with the H₁R active-state models based on the crystal structure of bovine rhodopsin, the E2-loop differs in its contact to the ligand bound in the binding pocket. In the bovine rhodopsin-based model, the backbone carbonyl of Lys187 (gpH₁R) interacts with large histaprodifens in the binding pocket, but in the hβ₂AR-based model, Lys187 (gpH₁R) is located distantly from the binding pocket. In conclusion, the differences in N terminus and E2-loop between hH₁R and gpH₁R exert an influence on affinity and/or potency for histamine and phenoprodifens 6R, 6R, and 6S.

G protein-coupled receptors (GPCRs) represent the largest class of cell-surface receptors, which consist of seven transmembrane helices that are connected by three extracellular and three intracellular loops (Kristiansen, 2004). The histamine H₁ receptor (H₁R) is a biogenic amine receptor that belongs to class I of the GPCRs (Foord et al., 2005), and it interacts with G proteins to activate phospholipase C (Hill et al., 1997).

In histaprodifens (Fig. 1), identified as potent H₁R agonists at the guinea pig (gp) ileum (Elz et al., 2000; Menghin et al., 2003), a 3,3-diphenylpropyl moiety is combined with a 2-substituted histamine. A pharmacological characterization of histaprodifens at the human H₁R (hH₁R) and guinea pig H₁R (gpH₁R) showed significant species differences (Seifert et al., 2003; Straßer et al., 2008). Several amino acids are involved in histamine binding: Asp²,³,³² (Ohta et al., 1994; Nonaka et al., 1998), Lys⁵,³⁹ (Leurs et al., 1995; Bruysters et al., 2004), Thr⁵,⁴² and Asn⁵,⁴⁶ (Leurs at al., 1994; Ohta et al., 1994), and Phe⁶,⁵⁵ (Bruysters et al., 2004). The amino acid side chains Trp⁴,⁵⁶, Lys⁵,³⁹, Phe⁶,⁵², and Phe⁶,⁵ were found to interact with H₁R agonists (Wieland et al., 1999; Gillard et al., 2002).

Asn²,⁶¹ acts as a selectivity switch between hH₁R and gpH₁R for suprahistaprodifen and dimeric histaprodifen (Bruysters et al., 2005). However, pharmacological analysis of suprahistaprodifen and dimeric histaprodifen at the bovine H₁R and the rat H₁R (Straßer et al., 2008) showed that the amino acid in position 2.61 cannot be exclusively responsible for the observed species differences.

Molecular modeling studies of dimeric histaprodifen in the binding pocket of the gpH₁R suggest that the second extracellular loop (E2-loop) is in close contact to dimeric histaprodifen.
prodifen and suprahistaprodifen and participates in binding of large ligands by forming hydrogen bonds (Straßer et al., 2008). Furthermore, the N terminus presumably interacts with the E2-loop. Comparing the amino acid sequences of the N terminus and E2-loop between hH1R and gpH1R, species differences of approximately 60% for the N terminus and of approximately 30% for the E2-loop are found (Fig. 2).

Several studies have analyzed the contribution of the E2-loop to agonist or antagonist binding or activation of GPCRs, and the same analysis was performed with the adenosine A1, A2a, A3 receptors (Olah et al., 1994; Kim et al., 1996), the dopamine D2 receptor (Shi and Javitch, 2004), the muscarinic acetylcholine M3 receptor (Scarselli et al., 2007), the α2A-adrenergic receptor (Laurila et al., 2007), and the histamine H2 receptor (Preuss et al., 2007). To study the influence of the species differences in N terminus and E2-loop between hH1R and gpH1R, we constructed chimeric hH1R with gp E2-loop (hgpE2H1R) and hH1R with gp N terminus and gp E2-loop (hgpNgpE2H1R) (Fig. 3A). The wild-type and chimeric H1Rs were coexpressed with the regulator of G protein signaling 4 (RGS4) in Sf9 insect cells. We characterized some histaprodifen (Fig. 1) in [3H]mepyramine competition binding assay and GTPase assay. In addition, active-state models based on the crystal structure of bovine rhodopsin and 2 receptor were constructed and compared to each other.

Materials and Methods

Materials. Phusion high-fidelity polymerase, all restriction enzymes, and T4 DNA ligase were obtained from New England Biolabs (Ipswich, MA). The anti-Flag IgG (M1 monoclonal antibody) was obtained from Sigma-Aldrich (St. Louis, MO), and the anti-RGS4 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [γ-32P]GTP was synthesized as described previously (Preuss et al., 2007), and [3H]mepyramine (30.0 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). A Rotiszint ecoplus (Roth, Karlsruhe, Germany) liquid scintillation cocktail was used. Histaprodifen were synthesized as described by Elz et al. (2000), Menghin et al. (2003), and Striegl (2006).

Construction of pGEMhgpE2H1R, pGEMhgpNgpE2H1R, pVLhgpE2H1R, and pVLhgpNgpE2H1R. First, a pGEM-3Z-SF-hgpNH1R plasmid was constructed. Therefore, pGEM-3Z-SF-gpH1R plasmid and pGEM-3Z-SF-hH1R plasmid were double-digested with HindIII and PflMI, resulting in the fragments h-I, h-II, gp-I, and gp-II (Fig. 3B). The fragments h-I and gp-I were digested with PvuII, producing four fragments as follows: h-Ia, h-Ib, gp-Ia, and gp-Ib (Fig. 3B). For generation of pGEM-3Z-SF-plasmid, the N terminus, and the very beginning of transmembrane domain (TM) I. After separation and cleaning of the fragments, a triple ligation with gp-Ia, h-Ib, and h-II was performed. For generation of pGEM-3Z-SF-hgpE2H1R and pGEM-3Z-SF-hgpNgpE2H1R, overlap-extension polymerase chain reactions (PCR) (PCR Ia, PCR Ib, and PCR II) with pGEM-3Z-SF-hH1R plasmid, respectively, as template were performed. In PCR Ia, the DNA fragment with the signal peptide (S), the FLAG epitope (F), and the first part of the hH1R up to transmembrane domain IV with guinea pig E2-loop was amplified. In PCR Ib, the DNA fragment encoding the guinea pig E2-loop, the second part of the hH1R, and the His6 tag (CCATCATGACATCAC) were annealed in the gp E2-loop-encoding region, resulting in PCR fragments that encode the SF, hgpE2H1R, the His6 tag, the stop codon, and an Xhol site. For PCRs, the following primers targeting the E2-loop were used:
GCTCACTCATTAGGCACC (forward, PCR Ia, PCR II); TTTCCTTCCGGGGGCTCAGCTCTAGTCGGGGCTCACTAGTCGGGGCCATGAAGTGGTG
CCAGCCTA- GAATGGGAATAAC (reverse, PCR Ia); CACCACTTCATGGCCCCG
- ACTAGTGAGCCCCGGGAGAAA
AAGTGTGAGACAGACTTCTAT (forward, PCR Ib); and GGATCCTCTAGATTAGTGATGGTGATG-
ATGGTG (reverse, PCR Ib, PCR II). The underlined code indicates a silent mutation for introduction of a unique diagnostic SpeI site. Italic letters designate the E2-loop. The resulting PCR fragment was double-digested with HindIII and XbaI and cloned into the pGEM-3Z plasmid using pGEM-3Z-SF gpH1R as a template. The sequences of hgpH1R and hgpNgpE2H1R, cloned into the pGEM-3Z-SF plasmid, were checked for their correctness by sequencing (Entelechon, Regensburg, Ger-
many). pGEM-3Z-SF-hgpE2H1R and pGEM-3Z-SF-hgpNgpE2H1R were used as a template to clone hgpE2H1R and hgpNgpE2H1R into the pVL1392 baculovirus transfer vector using the restriction sites BamHI and XbaI. All wild-type and chimeric H1Rs were N-terminally tagged with the signal peptide and FLAG epitope ATGAAGACGATCATCGCATCATGACGCC and C-terminally tagged with the His6 tag CATCATCATTACG.

Preparation of Compound Stock Solutions. Chemical structures of the analyzed compounds are given in Fig. 1. Compounds 1 and 8 (10 mM each) were dissolved in double-distilled water. Compounds 2 to 6 were dissolved in a solvent mixture containing 30% (v/v) dimethyl sulfoxide (DMSO), 30% (v/v) Tris-HCl, pH 7.4 (10 mM), and 40% (v/v) double-distilled water, 5 mM each. Compound 7 was dissolved in 50% (v/v) DMSO and 50% (v/v) Tris-HCl, pH 7.4 (10 mM), at a concentration of 1 mM. This lead to a 1:10 dilution of DMSO in each assay tube. Reference binding and GTPase assays with three preparations of histamine dissolved in 1) double-distilled water, 2) 30% (v/v) DMSO, 30% (v/v) Tris-HCl, pH 7.4 (10 mM), 40% (v/v) double-distilled water [equivalent to a final DMSO concentration of 3% (v/v)], and 3) 50% (v/v) DMSO, 50% (v/v) Tris-HCl, pH 7.4 (10 mM), equivalent to a final DMSO concentration of 5% (v/v), respectively, revealed only a decrease in the radioactivity counted, but no shift in pKi, and pEC50 values.

Miscellaneous. Cell culture, generation of recombinant baculoviruses, and membrane preparations were performed as described in Straßer et al. (2008). The determination of protein concentration, the SDS-polyacrylamide gel electrophoresis, and the immunoblot analysis were performed as described previously (Seifert et al., 2003; Straßer et al., 2008). The pharmacological assays, i.e., [3H]mepyramine saturation binding assay, [3H]mepyramine competition binding assay, and steady-state GTPase assay, were performed as described previously (Straßer et al., 2008). All assays for comparison of pharmacological data were performed as described previously (Seifert et al., 2003; Straßer et al., 2008). For all calculations, the software package GROMACS 3.3.1 (van der Spoel et al., 2004) was used in combination with the ffG53A6 force field (Oostenbrink et al., 2004). The force-field parameters for dimeric histaprodifen 7 were adopted from the ffG53A6 force field.

Results

Immunological Detection of the H1R Constructs. All H1R constructs were immunologically detected with the M1 antibody (Fig. 5). hH1R showed a strong band at ~85 kDa. In contrast, gpH1R showed a strong band at 25 kDa, intermediate bands at ~30 and ~36 kDa, and faint bands at ~50 and ~100 kDa. For hgpE2H1R, a strong band was detected at ~60 kDa.

Fig. 4. Alignment of the E2-loop of hH1R and gpH1R to the amino acid sequence of bovine rhodopsin and human β2-adrenergic receptor. The cysteine forming a disulfide bridge to a cysteine residue in transmembrane domain III is shaded in gray. Hyphens indicate missing amino acids.

Fig. 5. Immunological detection of hH1R, hgpE2H1R, hgpNgpE2H1R, and gpH1R expressed in SF9 insect cell membranes. SF9 cells, expressing one of the H1R isoforms and RGS4, were analyzed in an immunoblot as described under Materials and Methods. For immunological detection, the M1 antibody was used. Each line represents one of the four analyzed H1R constructs. Numbers at the left indicate the apparent molecular masses of the proteins in kilodaltons.
crease in p_i values was found in the series hH1R/H20851

total was determined in the presence of 10 

hgpE2H1R

nonspecific binding of [3H]mepyramine in Sf9 cells express-

forms, namely hH1R and gpH1R. These experimental results

may be explained with different N-glycosylation states. At

the N terminus, hH1R exhibits two and gpH1R exhibits one

glycosylation site(s). Homology models of H1Rs suggest that

there is one additional N-glycosylation site for hH1R in the

e2-loop, but not for gpH1R. Thus, hH1R exhibits three

(hgpE2H1R two, hgpNgpE2H1R one, and gpH1R one) glycosa-
ylation site(s).

Analysis of the H1R Wild-Type and Chimeric Species

Isoforms in the [3H]Mepyramine Saturation Binding

Assay. The K_D and B_max values, which were determined in

the [3H]mepyramine saturation binding assay, are given in

Table 1. There was no significant difference in the K_D values of

hH1R, hgpE2H1R, and hgpNgpE2H1R, which were 1.5 to

2-fold higher (p < 0.05) than at gpH1R. The B_max value for

hgpE2H1R was in the same range as the value for hH1R. The

B_max value for hgpNgpE2H1R was approximately 3-fold lower

(p < 0.05) than that of hgpE2H1R, corresponding to the weaker

immunoreactivity in the immunoblot (Fig. 5). The nonspecific binding of [3H]mepyramine in Sf9 cells express-
ing wild-type and chimeric species isoforms was maximally

30%.

Analysis of Histaprodifen at H1R Wild-Type and

Chimeric Species Isoforms in the [3H]Mepyramine

Competition Binding Assay. The affinities of compounds 1
to 7, determined in the competition binding assay, are given in

Table 2. For compounds 1 to 4S and 7, no significant differ-

cence in pK_i values was found between hH1R, hgpE2H1R,

and hgpNgpE2H1R. However, for phenoprodifen 5 as well as for

the chiral phenoprodifen 6R and 6S, a significant decrease in

pK_i values was found in the series hH1R > hgpE2H1R ≥ hgpNgpE2H1R

(Fig. 6). For compound 5 at hH1R, the pK_i value was significantly (p < 0.005) higher than at hgpE2H1R and hgpNgpE2H1R. At hH1R, the pK_i value of 6R was significantly lower than that of 6S (p < 0.05).

Constitutive Activity and Maximal Stimulation with

Histamine of hH1R, hgpE2H1R, hgpNgpE2H1R, and

gpH1R. The hH1R couples to endogenous G_q proteins of Sf9

insect cells (Houston et al., 2002). Agonist activation of the

G_q proteins is detected by an increase of high-affinity GTP

hydrolysis in membranes expressing H1R and RGS proteins.

The basal GTPase activity of the wild-type and chimeric H1Rs

were significant lower (p < 0.005) than at gpH1R. The

maximal stimulation at hH1R, hgpE2H1R, and gpH1R was

significantly lower than at hgpE2H1R (Savitz et al., 2008). In accordance with the

results for the competition binding (Fig. 6) assay, a decrease in pK_i values was found for 6R as well as for 6S in the series hH1R > hgpE2H1R > hgpNgpE2H1R (Table 4).

Binding Mode of Dimeric Histaprodifen in gpH1R

Models Based on Two Different Crystal Structures.

The binding mode of dimeric histaprodifen 7 docked into the

binding pocket of the gpH1R active-state model, based on the

crystal structure of bovine rhodopsin (Fig. 8A), was described

in detail in Straßer et al. (2008). In comparison, the molecu-

lar dynamics simulations with dimeric histaprodifen revealed

some differences in the resulting binding mode. The largest difference between the two active gpH1R models was found in the

conformational and flexibility (Fig. 9) of the E2-loop. In the

bovine rhodopsin-based gpH1R model, the part of the E2-loop that shows species differences between hH1R and gpH1R is in close contact to the upper part of the binding pocket, near the transmembrane domain II. It is interesting to note that the backbone carbonyl of Lys187 (gpH1R) forms a hydrogen bond to an imidazole moiety of dimeric histaprodifen. Because of a shift of the E2-loop in the hbetaAR-based model, Lys187 does

TABLE 1

[3H]Mepyramine saturation binding in Sf9 cell membranes expressing

hH1R, hgpE2H1R, hgpNgpE2H1R, and gpH1R with RGS5

Sf9 cell membranes coexpressing one of the H1Rs and RGS5 were incubated with 0.2

nM [3H]MEP as described under Materials and Methods. Nonspecific binding was
determined in the presence of 10 

M diphenhydramine and was subtracted from total [3H]MEP binding. The resulting binding data were analyzed by nonlinear

regression and were best fitted to monophasic saturation curves. Data shown are the

means ± S.E.M. of three independent membrane preparations, each one analyzed in

tripllicate.

<table>
<thead>
<tr>
<th>Compound</th>
<th>K_D (nM)</th>
<th>B_max (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hH1R</td>
<td>4.49 ± 0.05</td>
<td>5.85 ± 1.67</td>
</tr>
<tr>
<td>hgpE2H1R</td>
<td>4.17 ± 0.75</td>
<td>3.91 ± 0.73</td>
</tr>
<tr>
<td>hgpNgpE2H1R</td>
<td>3.77 ± 0.67</td>
<td>1.90 ± 0.42</td>
</tr>
<tr>
<td>gpH1R</td>
<td>2.53 ± 0.23</td>
<td>3.94 ± 0.83</td>
</tr>
</tbody>
</table>
TABLE 2

Affinities of histamine and histaprodifens at hH1R, hgpE2H1R, hgpNgpE2H1R, and gpH1R coexpressed with RGS4 in Sf9 cell membranes in the equilibrium competition binding assay

<table>
<thead>
<tr>
<th>Cpd</th>
<th>hH1R</th>
<th>hgpE2H1R</th>
<th>hgpNgpE2H1R</th>
<th>gpH1R</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.62 ± 0.03</td>
<td>5.74 ± 0.04</td>
<td>5.75 ± 0.12</td>
<td>5.50 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>6.47 ± 0.11</td>
<td>6.21 ± 0.08</td>
<td>6.08 ± 0.08</td>
<td>6.38 ± 0.11</td>
</tr>
<tr>
<td>3</td>
<td>6.33 ± 0.09</td>
<td>6.23 ± 0.03</td>
<td>6.29 ± 0.10</td>
<td>7.11 ± 0.10</td>
</tr>
<tr>
<td>4R</td>
<td>6.14 ± 0.07</td>
<td>5.96 ± 0.09</td>
<td>6.00 ± 0.07</td>
<td>6.94 ± 0.08</td>
</tr>
<tr>
<td>4S</td>
<td>5.40 ± 0.09</td>
<td>5.53 ± 0.08</td>
<td>5.58 ± 0.06</td>
<td>6.12 ± 0.08</td>
</tr>
<tr>
<td>5</td>
<td>6.60 ± 0.07</td>
<td>5.92 ± 0.04</td>
<td>6.01 ± 0.02</td>
<td>7.33 ± 0.08</td>
</tr>
<tr>
<td>6R</td>
<td>6.08 ± 0.06</td>
<td>5.94 ± 0.06</td>
<td>5.72 ± 0.08</td>
<td>6.74 ± 0.02</td>
</tr>
<tr>
<td>6S</td>
<td>6.40 ± 0.15</td>
<td>6.04 ± 0.11</td>
<td>5.76 ± 0.04</td>
<td>6.38 ± 0.17</td>
</tr>
<tr>
<td>7</td>
<td>6.67 ± 0.01</td>
<td>6.37 ± 0.08</td>
<td>6.58 ± 0.18</td>
<td>7.33 ± 0.14</td>
</tr>
</tbody>
</table>

a Comparison with pKᵢ at hH1R, p < 0.005.
b Comparison with pKᵢ at hH1R, p < 0.05.
c Comparison with pKᵢ at hH1R, p < 0.005; comparison with pKᵢ at hgpE2H1R, p < 0.05.

not interact with dimeric histaprodifen. The following amino acids were found to participate in the binding of T7 in the new model: Leu39 (TM1, 1.35), Leu43 (TM1, 1.39), Leu97 (TM2, 2.65), Trp112 (TM3, 3.28), Ile124 (TM3, 3.40), Trp429 (TM6, 6.48), and Trp456 (TM7, 7.40). The quaternary amine moeity in the center of 7 interacts electrostatically with the negatively charged Asp116 (TM3, 3.32) and Tyr459 (TM7, 7.43). One of the imidazole moieties forms stable hydrogen bonds

with Ser120 (TM3, 3.36) and Tyr432 (TM6, 6.51). The second imidazole moiety forms stable hydrogen bonds to Glu190 (E2-loop) and Trp456 (TM7, 7.40) (Fig. 8). Both dipenyl propyl moieties of the dimeric histaprodifen are embedded in hydrophobic pockets.

The conserved disulfide bond between two cysteine side chains in TM3 and the E2-loop is responsible for a reduced flexibility in this region of the E2-loop (Fig. 9). However, although molecular dynamic simulations show a high flexibility of the nonrestricted parts of the E2-loop, a movement toward the binding pocket is notably observed. The interaction between the conserved Glu and the ligand is observed only in cases where the E2-loop is close to the binding pocket. This hydrogen bond stabilizes the conformation of the ligand in the binding pocket and reduces the flexibility of the ligand. The exchange of N terminus and E2-loop exerted an influence onto the hydrogen bond network between N terminus, E1-loop, and E2-loop with an effect on E2-loop conformation and flexibility (Fig. 9).

Discussion

GTPase Activation and Potency of Histamine at the Chimeric hgpNgpE2H1R Compared with hH1R. Our steady-state GTPase assay data show that the double-chimeric hgpNgpE2H1R has influence on the maximal Gq₃₆-protein stimulation with histamine and potency of histamine, compared with hH1R. Because the single-chimeric hgpE2H1R showed no influence on the maximal Gq₃₆-protein stimulation with histamine compared with hH1R, it can be concluded that the N terminus or the N terminus in combination with the E2-loop is responsible for this observation. Molecular dynamics simulations revealed that the exchange of N terminus and E2-loop has an influence on the hydrogen bond network between N terminus, E1-loop, and E2-loop with an effect on E2-loop conformation and flexibility (Fig. 9).

Influence of the N Terminus and E2-Loop on Pharmacological Differences of Histaprodifens between hH1R and gpH1R. The experimental data show that the chimeric
Analysis of the effects of histamine and mepramine and determination of the constitutive activity of hH1R, hgpE2H1R, hgpNgpE2H1R, and gpH1R in the steady-state GTPase assay

Sf9 cell membranes expressing hH1R, hgpE2H1R, hgpNgpE2H1R, or gpH1R in combination with RGS4 were used to study the constitutive activity. GTPase assays were performed as described under Materials and Methods. The concentration-response curves of the inverse agonist MEP (4S) were determined in a concentration range from 0.1 to 10 μM. Data were analyzed by nonlinear regression and were best fit to sigmoidal concentration-response curves. The efficacy of histamine was set 1.00. The ΔHA value refers to the difference of basal activity relative to GTP hydrolysis in presence of 10 μM MEP. The relative effect of MEP in the last column is the percentage effect of MEP relative to HA. Data shown are means ± S.E.M. of three experiments, each one performed in duplicate.

<table>
<thead>
<tr>
<th>Cpd</th>
<th>hH1R</th>
<th>hgpE2H1R</th>
<th>hgpNgpE2H1R</th>
<th>gpH1R</th>
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<tbody>
<tr>
<td></td>
<td>pEC50</td>
<td>E max</td>
<td>pEC50</td>
<td>E max</td>
</tr>
<tr>
<td>hH1R</td>
<td>8.24 ± 0.26</td>
<td>2.17 ± 0.38</td>
<td>0.37 ± 0.17</td>
<td>0.11 ± 0.01</td>
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<tr>
<td>hgpE2H1R</td>
<td>7.75 ± 0.21</td>
<td>2.41 ± 0.21</td>
<td>0.62 ± 0.17</td>
<td>0.07 ± 0.01</td>
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<tr>
<td>hgpNgpE2H1R</td>
<td>7.92 ± 0.27</td>
<td>4.06 ± 0.29</td>
<td>2.15 ± 0.63</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>gpH1R</td>
<td>8.86 ± 0.18</td>
<td>1.89 ± 0.26</td>
<td>0.48 ± 0.17</td>
<td>0.26 ± 0.03</td>
</tr>
</tbody>
</table>

N Terminus and E2-Loop in H1-Receptor Isoforms

Our molecular dynamics simulations with the new gpH1R model, based on the crystal structure of hβ2AR, show that the backbone carbonyl of Lys187 (gpH1R) is far away from the binding pocket and does not interact with a large histaprodifen in the binding pocket, as suggested by the gpH1R model based on the crystal structure of bovine rhodopsin. In addition, the molecular dynamic simulations based on the hβ2AR model show that the highly conserved Glu181 (hH1R) and Glu190 (gpH1R), respectively, within the H2R family point toward the binding pocket with formation of a stable hydrogen bond to the ligand and point away from the binding pocket forming a stable hydrogen bond to Tyr96 (TM2, 2.42). The MD simulations have shown that the exchange of the N terminus and E2-loop exhibits an influence of the hydrogen bond network in the extracellular part of the receptor with an effect on E2-loop conformation and flexibility, which results in decreased pKd and pKB values for the phenoprodifen 5, 6R, and 6S at the chimeric H1R. Furthermore, we assume that 5, 6R, and 6S can bind in two different orientations into the binding pocket (Straler et al., 2008). It is possible that a change in orientation of 5, 6R, and 6S in the series hH1R – hgpE2H1R – hgpNgpE2H1R can explain our experimental data. Because the largest differences between hH1R, the chimeric H1Rs, and gpH1R occur in the extracellular surface of the receptor, it is assumed that this possible change in orientation is determined kinetically during the early steps of the ligand binding process. To test this hypothesis, sophisticated kinetic binding studies have to be carried out. Furthermore, extensive molecular dynamics simulations with compounds...
at wild-type and chimeric H1Rs are required to quantify the question of orientation.

Our new H1R models indicate that the region of the E2-loop with species differences in amino acid sequence is pointing away from the binding pocket. It is possible that highly conserved amino acids in the E2-loop of H1Rs are involved in ligand binding, e.g., Glu190 (E2-loop, gpH1R), which shows electrostatic interaction with dimeric histaprodifen in the old gpH1R model as well as in the new model. Additional experimental studies will be carried out to analyze the participation of Glu190 (E2-loop, gpH1R) in binding of large histaprodifen.

Role of the Extracellular Loop E2 in Ligand Binding and Activation of GPCRs. Compared with other GPCR regions, relatively little attention has been paid thus far to the contribution of the second extracellular loop E2 in ligand recognition and ligand binding in biogenic amine and nucleoside GPCRs. Glutamate residues in the E2-loop of the human A2a adenosine receptor are indirectly or directly involved in ligand binding (Kim et al., 1996). For the 1-adrenergic receptor, amino acids, located in the extracellular loop E2, play a role in subtype-selective antagonist binding (Zhao et al., 1996). In addition, the affinity of yohimbine to the 2A-adrenergic receptor is significantly influenced by interactions with the extracellular loop E2 (Laurila et al., 2007). Moreover, specific residues of the E2-loop are directly involved in forming the binding pocket of the dopamine D2 receptor (Shi and Javitch, 2004). For the muscarinic M3 receptor, several amino acids in the E2-loop are important for efficient agonist-induced activation of the muscarinic M3 receptor (Scarselli et al., 2007). In contrast to these results, the E2-loop does not contribute to the species selectivity of guanidinetype agonists at human and guinea pig histamine H2 receptor (Preuss et al., 2007). Our present study shows that the E2-loop and the E2-loop in combination with the N terminus have ligand-specific influence on the pharmacology of the H1R. Based on these data, it can be concluded that the extracellular loop E2 as well as the N terminus contribute to ligand binding, receptor activation, and selectivity in biogenic amine and nucleoside GPCRs. Therefore, it seems to be

Fig. 7. Concentration-response curves for histamine 1 at hH1R, hgpE2H1R, and hgpNgpE2H1R in the steady-state GTPase assay. The experiments were performed using Sf9 cell membranes coexpressing hH1R, hgpE2H1R, or hgpNgpE2H1R and RGS4 as described under Materials and Methods. The downward-facing triangle (▼) shows the data for hH1R, and the square (■) shows the data for the chimerical hgpNgpE2H1R. Data shown are the means ± S.E.M. of at least three experiments, each one performed in duplicate with independent membrane preparations.

Fig. 8. Binding mode of dimeric histaprodifen 7 in the active gpH1R model. Snapshot of dimeric histaprodifen 7 in the binding pocket of the active gpH1R model during MD simulation. A, model based on the crystal structure of bovine rhodopsin. B, model based on the crystal structure of the h1AR. MD simulations were performed as described under Materials and Methods. Hydrogen bonds and electrostatic interactions between ligand and amino acids are marked as red dashed lines.

Fig. 9. Root mean square (rms) fluctuation of the backbone atoms of the E2-loop. The label Cys-Cys indicates the position of the disulfide bridge between E2-loop and TM3. This part of the E2-loop, which shows large species differences and is in no contact with the binding pocket, exhibits a decrease in backbone fluctuation within the series hH1R > hgpNgpE2H1R > gpH1R (region between the labels E2-start and Cys-Cys). The region of the E2-loop with no species differences and contact to the ligand in the binding pocket shows a higher fluctuation for the hgpNgpE2H1R compared with the wild-type H1R. For all species, the fluctuation is reduced in the region of the disulfide bridge (Cys-Cys). All calculations were performed with GROMACS 3.3.1.
N Terminus and E2-Loop in H<sub>1</sub>-Receptor Isoforms


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