Molecular and Cellular Analysis of Aminergic G Protein-Coupled Receptors: Histamine H_2, H_4 and β_2-Adrenergic Receptors, a Scientific Paradigm

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)
der Naturwissenschaftlichen Fakultät IV – Chemie und Pharmazie –
der Universität Regensburg

vorgelegt von

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im Jahr 2011

Die vorliegende Arbeit entstand unter der Leitung von Herrn Prof. Dr. A. Buschauer und Herrn Prof. Dr. R. Seifert im Zeitraum von Oktober 2008 bis November 2011 am Institut für Pharmazie der Naturwissenschaftlichen Fakultät IV – Chemie und Pharmazie – der Universität Regensburg und am Institut für Pharmakologie der Medizinischen Hochschule Hannover.

Promotionsgesuch wurde eingereicht im November 2011.
Tag der mündlichen Prüfung: 09. Dezember 2011

Prüfungsausschuss:

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Danksagungen

Ich möchte mich ganz herzlich bei allen bedanken, die zum Gelingen dieser Arbeit beigetragen haben und mich während der Promotionszeit begleitet haben.

Ein besonderer Dank gilt Herrn Prof. Dr. Armin Buschauer und Herrn Prof. Dr. Roland Seifert, dass sie mir gemeinsam die Gelegenheit gegeben haben, an diesem interessanten Projekt zu arbeiten. Beiden möchte ich für die höchst kompetente Unterstützung und die stets konstruktive Kritik ausdrücklich danken. Herrn Buschauer danke ich zudem für seine Förderung. Herrn Seifert möchte ich besonders für die Möglichkeit danken, am Institut für Pharmakologie der Medizinischen Hochschule Hannover als Gastwissenschaftlerin arbeiten zu dürfen und für die gute Betreuung trotz der großen Entfernung.

Desweiteren danke ich

Frau Dr. Andrea Straßer für die unentbehrliche Unterstützung und nützliche fachliche Ratschläge, insbesondere zu Beginn meiner Promotion, und für das Molecular Modelling zum H_4 Rezeptor,

Herrn Prof. Dr. Jens Schlossmann für die Möglichkeit, am Lehrstuhl für Pharmakologie und Toxikologie den Großteil meiner Experimente durchführen zu können,

Herrn Prof. Dr. Günther Bernhardt für seine wissenschaftlichen Anregungen sowie für seinen hilfreichen Beitrag bei der Durchsicht dieser Arbeit,

Herrn Dr. David Schnell für die Bereitstellung seiner Bindungsdaten am humanen und canin H_4 Rezeptor sowie für die praktische Einführung in die Durchführung der GTPase Assays,

Herrn Dr. Tobias Birnkammer für die freundliche Bereitstellung der bivalenten Liganden,

Frau Gertraud Wilberg für die Vorbereitung der Sf9 Zellen, die Einarbeitung in die SDS-PAGE und Western Blot und für ihre Hilfsbereitschaft sowie Frau Maria Beer-Krön und Frau Sabine Dirrigl für die Hilfe bei der Durchführung der GTPase Assays und der Bindungsexperimente,

Frau Rita Prenzyna, Frau Martina Wechler, Frau Silvia Heinrich, Frau Karin Reindl und Frau Uta Hasselmann für die stete Bereitschaft bei allen organisatorischen Angelegenheiten zu helfen,

den „Histamin-Gruppen“ aus Regensburg und Hannover für die anregenden wissenschaftlichen Diskussionen und für die engagierte Zusammenarbeit,

Herrn Prof. Dr. Frieder Kees für sein Interesse am Fortgang meiner Arbeit, für seine Geduld beim Korrekturlesen meiner deutschen Texte und für seinen guten schwarzen Tee,

der Deutschen Forschungsgemeinschaft für die finanzielle Förderung in Rahmen des Graduiertenkollegs GRK 760.

Am Institut für Pharmakologie der Medizinischen Hochschule Hannover danke ich

Frau Solveig Kälble für ihre tatkräftige Unterstützung bei der Bestimmung der H_2R Isoformen an neutrophilen Granulozyten sowie für die Durchführung von Superoxid Anion und cAMP Experimenten auch nach meiner Rückkehr nach Regensburg,

Herrn Prof. Dr. Volkhard Kaever für seine Hilfestellung bezüglich der Massenspektrometrie,

Frau Dr. Heike Burhenne für die Durchführung der Messungen am Massenspektrometer und ihre kompetente Hilfe diesbezüglich,

Herrn Michael Reinartz für die Zusammenarbeit bei Experimenten an neutrophilen Granulozyten,

Frau Juliane von der Ohe für die Aufnahme der Mikroskopbilder,

Frau Annette Stanke für die Hilfe bei den bürokratischen Angelegenheiten,

allen anderen Institutsmitgliedern, die mir auf irgendeine Art und Weise geholfen haben.

Meinen Kolleginnen und Kollegen am Lehrstuhl für Pharmazeutische/Medizinische Chemie II Stefanie Bauer, Paul Baumeister, Dr. Tobias Birnkammer, Dr. Daniela Erdmann, Dr. Miriam Ertel, Johannes Felixberger, Roland Geyer, Janina Hamberger, Tobias Holzammer, Stefan Huber, Dr. Patrick Igel, Nicole Kagermeier, Melanie Kaske, Katerina Ladova, Dr. Miroslaw Lopuch, Carolin Meyer, Uwe Nordemann, Dr. Nikola Pluym, Dr. Nathalie Pop, Christian Textor, Dr. Oksana Sereda, Laura Waltl und anderen danke ich für ihre Kollegialität sowie für die angenehme Zeit an und außerhalb der Universität.

Ein Dankeschön geht an die ehemaligen „Seifert-Doktoranden“ aus Regensburg Dr. Heidrun Appl, Dr. Miriam Erdorf, Dr. Martin Göttle, Dr. Melanie Hübner, Dr. Erich Schneider, Dr. David Schnell und Dr. Sarah Sutor für ihre Hilfe im Laboralltag und für die schöne gemeinsame Zeit. Vor allem David, Erich und Heidrun haben mir bei vielen wissenschaftlichen und technischen Fragen weitergeholfen. Weiterhin danke ich meinen Kolleginnen und Kollegen am Lehrstuhl für Pharmakologie und Toxikologie Dr. Mathias Desch, Dr. Bernhard Hieke, Johannes Hüttner, Franziska Limmer, Petra Müller-Wille, Katharina Salb, Dr. Elisabeth Schinner, Andrea Schramm, Julia Thoma und Stefanie Wolferstetter für das angenehme Arbeitsklima. Besonderer Dank gilt Katharina, Lisa und Melanie für die aufbauenden Diskussionen, zahlreichen Joggingrunden und ihre Freundschaft.

Bei meinen lieben Nachbarn und ehemaligen Mitbewohnern Axel, Andi, Erika, Florian, Gunter, Katrin, Lutz, Moni, Tini, Volker und anderen möchte ich mich für die vielen lustigen und schönen gemeinsamen Momente in Regensburg bedanken.

Nina, Simona und Vanja möchte ich ganz herzlich für die langjährige Freundschaft danken.

Ein großes Dankeschön gilt meinen Eltern, meinen Großeltern, meiner Schwester Mojca mit Familie und meinem Bruder Marko, die immer für mich da waren und mir den nötigen Rückhalt gegeben haben. Hvala vam za vse!

An der letzten Stelle danke ich von ganzem Herzen meinem Freund Helmut, der mir geduldig und verständnisvoll während der Promotion zur Seite stand und mich mit voller Kraft unterstützte.

Contents

Abbreviations

5-HT	5-hydroxytriptamine
5-MHA	5-methylhistamine
aa	amino acid
AC	adenylyl cyclase
ADR	adrenaline
AMT	amthamine
ANOVA	analysis of variance
ARP	arpromidine
ATP	adenosine 5`-triphosphate
B_{max}	the maximal specific binding of a ligand
bp	base pair(s)
BRET	bioluminescence resonance energy transfer
c	canine
cAMP	cyclic adenosine 3`,5`-monophosphate
cDNA	complementary deoxyribonucleic acid
Ci	curie
CNS	central nervous system
CRE	cAMP response element
CREB	cAMP response element binding protein
DAG	1,2-diacylglycerol
DCI	dichloroisoproterenol
DIM	dimaprit
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOB	dobutamine
DPBS	Dulbecco's phosphate buffered saline
E1-, E2-, E3-loop	first, second, third extracellular loops of a G protein-coupled receptor
EC_{50}	agonist concentration which induces 50 % of the respective maximum effect
EDTA	ethylenediaminetetraacetic acid
E_{max}	efficacy (maximal response)
EPH	ephedrine
ERK	extracellular signal-regulated kinase

FAM	famotidine
FLAG	octapeptide epitope for the labeling of proteins
fMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
FPR	formyl peptide receptor
FRET	fluorescence resonance energy transfer
GAIP	regulator of G protein signalling 19
GDP	guanosine 5'-diphosphate
GEF	guanine nucleotide exchange factor
gp	guinea pig
GPCR	G protein-coupled receptor (synonymous term for seven transmembrane receptor)
$gpH_2R\text{-}G_{s\alpha S}$	fusion protein of the guinea pig H_2R and the short splice variant of $G\alpha_s$
GRK	G protein-coupled receptor kinase
GTP	guanosine 5`-triphosphate
$GTP\gamma S$	guanosine 5`-[γ-thio]triphosphate
$G\alpha_i$	α-subunit of G proteins that inhibits certain isoforms of adenylyl cyclase
$G\alpha_q$	α-subunit of G proteins that stimulates phospholipase C
$G\alpha_s$	α-subunit of G proteins that stimulates adenylyl cyclase
$G_{\alpha sS}$, $G_{\alpha sL}$	short and long splice variant of the G protein $G\alpha_s$
$G\beta\gamma$	βγ-subunits of a heterotrimeric G protein
h	human
H_1R, H_2R, H_3R, H_4R	histamine receptor subtypes
HA	histamine
$h_{cE1}H_4R$	human histamine H_4R with canine first extracellular loop
$h_{cE2}H_4R$	human histamine H_4R with canine second extracellular loop
$h_{cE3}H_4R$	human histamine H_4R with canine third extracellular loop
$h_{cNT}H_4R$	human histamine H_4R with canine N-terminus
$h_{gpNT}H_2R\text{-}G_{s\alpha S}$	human histamine H_2R with guinea pig N-terminus, fused to the short splice variant of $G\alpha_s$
$hH_2R\text{-}G_{s\alpha S}$	fusion protein of the human H_2R and the short splice variant of $G\alpha_s$
His_6	hexahistidine tag
HPLC	high performance (pressure) liquid chromatography
HPLC-MS/MS	high performance (pressure) liquid chromatography/tandem mass spectrometry

HR-MS	high resolution mass spectroscopy
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	antagonist concentration which reduces the effect of an agonist by 50 %
IgG	immunoglobulin G
IMP	impromidine
IP_3	inositol-1,4,5-trisphosphate
ISO	isoproterenol
K_B	inhibition constant of an antagonist/inverse agonist calculated from functional assays
K_D	equilibrium dissociation constant calculated from radioligand saturation binding assays
kDa	kilodalton
K_i	dissociation constant derived from a competition binding assay
LH	luteinizing hormone
LT	leukotriene
MAPK	mitogen-acitivated protein kinase
MD	molecular dynamics
MEP	mepyramine
min	minute(s)
mRNA	messenger ribonucleic acid
MS	mass spectrometry
NA	noradrenaline
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
N^G	guanidino-nitrogen
$O_2^{\bullet-}$	superoxide anion
pA_2	negative decadic logarithm of the concentration of antagonist that causes a concentration ratio of agonists of r = 2
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
pEC_{50}	negative decadic logarithm of the EC_{50} value
P_i	inorganic phosphate
PI3K	phosphatidylinositol-3-kinase
pIC_{50}	negative decadic logarithm of the IC_{50} value
PIP_2	phosphatidylinositol-4,5-bisphosphate

PKA	protein kinase A
pK_B	negative decadic logarithm of the K_B value
PKC	protein kinase C
PKD	protein kinase D
pK_i	negative decadic logarithm of the K_i value
PLC	phospholipase C
PTX	pertusus toxin
R	inactive state of a G protein-coupled receptor
R*	active state of a G protein-coupled receptor
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcription
S.E.M.	standard error of the mean
SAL	salbutamol
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sf9	insect cell line of *Spodoptera frugiperda*
SNP	single nucleotide polymorphism
SRM	selected reaction monitoring
THIO	thioperamide
TIO	tiotidine
TM	transmembrane domain of a G protein-coupled receptor
TM I-VII	numbering of transmembrane domains of a G protein-coupled receptor
t_R	retention time
Tris	tris(hydroxymethyl)aminomethan
ZOL	zolantidine
α_1AR, α_2AR	α-adrenergic receptor subtypes
β_1AR, β_2AR, β_3AR	β-adrenergic receptor subtypes

Author's Declaration

The author declares that the following work presented in this thesis was written by none other than himself. **Chapter 5** was written in the format of original publication. Apart from were indicated all work was performed or supervised by the author.

Molecular dynamic simulation described in **Chapter 5.4.7/5.5.4** and **Fig. 5.7** was performed by Dr. A. Strasser (Department of Pharmaceutical/Medicinal Chemistry II, University of Regensburg, Germany).

Chapter 1

General Introduction

1.1 The G protein-coupled receptor (GPCR) family

GPCRs are the largest group of membrane-integrated receptors (Lagerström and Schioth, 2008). The main structural characteristics of GPCRs are seven hydrophobic transmembrane domains forming α helices connected with three extracellular and three intracellular loops and terminated with an extracellular N-terminus and an intracellular C-terminus (Kobilka, 2007). The name of GPCRs originates from their ability to interact with guanine nucleotide-binding proteins (G proteins). However, especially in the newer literature these receptors are frequently referred to as seven transmembrane receptors (7TMRs) because of their structure and their ability to activate not only G protein-dependent but also G protein-independent signaling pathways (cf. section 1.3.1) (Kenakin and Miller, 2010). More than 800 full length members of the GPCR family have been identified in the human genome (Fredriksson *et al.*, 2003). GPCRs play important roles in numerous physiological and pathophysiological processes. About 30 % of all drugs currently available on the market target GPCRs (Overington *et al.*, 2006), which are continued to be explored as a very attractive and promising drug targets.

On the basis of phylogenetic criteria, the GPCR superfamiliy is divided into five main groups termed rhodopsin, glutamate, adhesion, frizzled/taste 2 and secretin receptor families (Lagerström and Schioth, 2008). The rhodopsin family, comprising about 700 members, is the largest family of GPCRs and is subdivided into four groups (α, β, γ and δ) (Lagerström and Schioth, 2008). The α group of rhodopsin-like GPCRs includes, among others, receptors that can be activated by biogenic amines. Receptors of interest in this thesis, i.e. the histamine H_2 receptor (H_2R), the histamine H_4 receptor (H_4R) and the β_2-adrenergic receptor (β_2AR), are members of this aminergic receptor cluster (Fredriksson *et al.*, 2003).

1.2 G protein-dependent signaling of GPCRs

In case of G protein-mediated signaling, the first event after binding of endogenous or synthetic agonists to GPCR is an interaction of the agonist-receptor complex with regulatory heterotrimeric G proteins consisting of an α subunit (Gα) and a βγ complex (Gβγ). Binding of an agonist to the GPCR stabilizes an active receptor conformation, which enables the dissociation of guanosine 5'-diphosphate (GDP) from the catalytically inactive Gα protein (Fig. 1.1, step 1). This allows the formation of a ternary complex consisting of an agonist, the receptor and the nucleotide-free heterotrimeric G protein (Fig. 1.1, step 2). For a given receptor agonist affinity is the highest, when the ternary complex is formed. Upon binding of guanosine 5`-triphosphate (GTP) to Gα, the ternary complex is disrupted resulting in reduced agonist affinity and dissociation of heterotrimeric G protein into the Gα and the Gβγ subunits (Fig. 1.1, step 3). Both the Gα-GTP and the Gβγ complex can activate downstream signaling

pathways (step 4). The intrinsic GTPase activity of the Gα subunit terminates G protein activation by the hydrolysis of GTP into GDP and inorganic phosphate (P_i) (Fig. 1.1, step 5). Then, Gα and Gβγ re-associate, and the initial state is restored (Fig. 1.1, step 6) (Hermans, 2003; Schneider and Seifert, 2010). Particularly noteworthy is the fact that some GPCRs are present in an active conformation and couple to G proteins in the absence of a bound agonist. This phenomenon is referred to as constitutive activity (Seifert and Wenzel-Seifert, 2002). The most common approaches applied in the investigation of GPCR activity at the level of the G protein cycle are (I) determination of high-affinity agonist binding to a given GPCR (e.g., using fluorescent or radio-labeled ligands), (II) monitoring of the dissociation of the ternary complex with e.g. radio-labeled non-hydrolysable GTP analogs like guanosine 5`-[γ-thio]triphosphate ([^{35}S]GTPγS binding assay) and (III) determination of the intrinsic GTPase activity of the Gα subunit by, e.g., measuring the hydrolysis of radio-labeled GTP to GDP and P_i in steady-state GTPase activity assays (Schneider and Seifert, 2010).

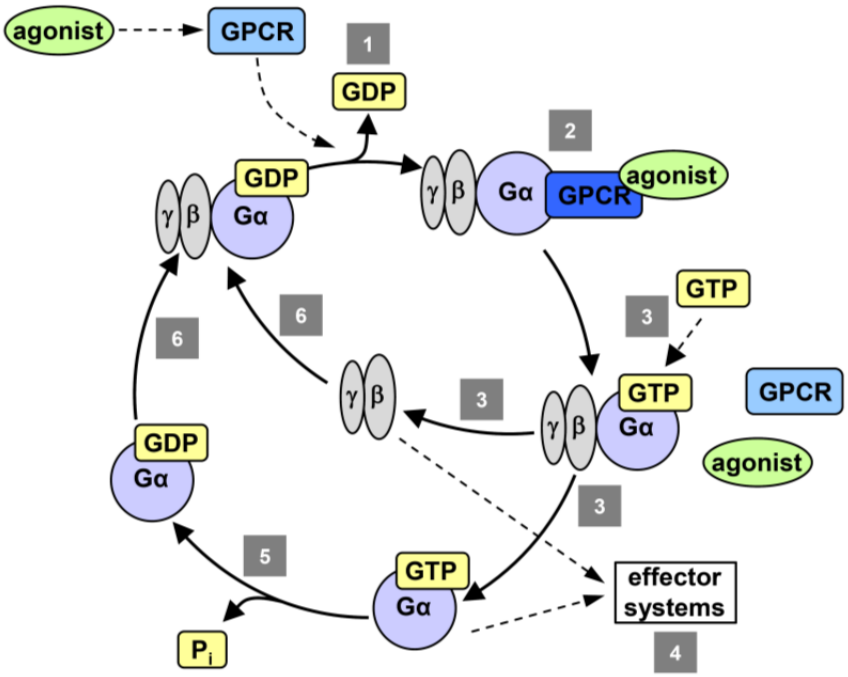


Fig. 1.1 G protein-activation by GPCRs upon agonist binding. Adapted from Schneider and Seifert (2010).

In order to describe pharmacological properties of a given GPCR upon interaction with an appropriate ligand, the extended ternary complex model is often used. According to this model, GPCRs exist in an inactive (R) and active (R*) conformation, which are equilibrated (Kenakin, 2004). Only GPCRs in the R* state are able to couple to G proteins and trigger signaling events. Agonists are ligands with higher affinity to the R* state, resulting in an activation or enhancement of GPCR-mediated downstream signaling events. On

contrary, inverse agonists are compounds that effectively stabilize the R state. Neutral antagonists bind to both states with comparable affinity and thereby compete with agonists and inverse agonists for binding to the receptor without affecting the equilibrium. Partial agonists have reduced ability to activate GPCRs, whereas partial inverse agonists have reduced ability to inhibit GPCRs relative to full agonists and inverse agonists, respectively (Seifert and Wenzel-Seifert, 2002).

GPCRs can couple to a diversity of heterotrimeric G proteins resulting in different intracellular signaling processes as shown in Table 1.1. Moreover, GPCRs can promiscuously interact with unrelated G proteins resulting in simultaneous activation of multiple signaling pathways (cf. section 1.3.2) (Hermans, 2003). Experimental approaches to the investigation of the G protein-coupling include the use of pertussis toxin (PTX) for selective inactivation of $G\alpha_{i/o}$ proteins (Vallar *et al.*, 1990; Gailly *et al.*, 2000), specific antibodies raised against G proteins (Kühn *et al.*, 1996; Alberts *et al.*, 2000; Cussac *et al.*, 2002), genetic repression (Tang *et al.*, 1995) and fusion protein approaches, where the C-terminus of the receptor is directly fused to the G protein of interest (Milligan, 2000; Wenzel-Seifert *et al.*, 2001).

Table 1.1 G-protein subunits and their effectors.

Subunit	Family	Subtypes	Effector(s)
α	α_s	$G\alpha_s$, $G\alpha_{olf}$	AC ↑
	$\alpha_{i/o}$	$G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$	AC ↓
		$G\alpha_{oA}$, $G\alpha_{oB}$	K^+ channels ↑
		$G\alpha_{t1}$, $G\alpha_{t2}$	PDE ↑
		$G\alpha_z$	PDE ↑, AC ↓
	$\alpha_{q/11}$	$G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14\text{-}16}$	PLC ↑
	$\alpha_{12/13}$	$G\alpha_{12}$, $G\alpha_{13}$	Rho guanine-nucleotide-exchange factors ↑
β and γ	$\beta_{1\text{-}5}$ and $\gamma_{1\text{-}12}$	various βγ complexes	AC ↑/↓, PLC ↑, PI3K ↑, PKC and PKD ↑, GPCR kinases ↑, Ca^{2+} and K^+ channels

Adapted from Hermans (Hermans, 2003), Worzfeld *et al.* (Worzfeld *et al.*, 2008) and Smrcka (Smrcka, 2008). AC, adenylyl cyclase; PDE, phosphodiesterase; PLC, phospholipase C; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; PKD, protein kinase D.

In addition to the investigation of the G protein cycle, compounds targeting GPCRs can be characterized by monitoring numerous downstream signaling events. A frequently used read-out is the measurement of cyclic adenosine 3',5'-monophosphate (cAMP) turnover as a result of an activation or inhibition of adenylyl cyclases (ACs) by G_s and G_i proteins, respectively. Moreover, determination of PLC-mediated inositol-1,4,5-trisphosphate (IP_3) formation or intracellular Ca^{2+} concentration are common methods for the assessment of G_q-coupled GPCRs (Schneider and Seifert, 2010). The gene reporter assay is another common

technique applied in the characterization of compounds in recombinant test systems (Lim *et al.*, 2008).

Termination of GPCR activation is achieved by an auto-regulatory process of desensitization. Desensitization processes can differ between different GPCRs (Evans *et al.*, 2010). Therefore, the desensitization of the β_2AR, one of the best characterized GPCRs, is described briefly in the following. Deactivation of the β_2AR after stimulation with an agonist occurs within minutes by phosphorylation of the receptor by PKA or G protein-coupled receptor kinases (GRKs) (Johnson, 1998). Sites of phosphorylation are serine and threonine residues in the third intracellular loop and the C-terminus of the receptor (Johnson, 2006). Phosphorylation of the β_2AR leads to uncoupling of the receptor from G proteins and to association with the scaffolding proteins β-arrestin-1 and/or β-arrestin-2. β_2AR/arrestin complexes are subsequently internalized into clathrin-coated pits, forming coated vesicles which then fuse with endosomes (Evans *et al.*, 2010). The internalized β_2ARs are either recycled back to the cell membrane or degraded (Hanyaloglu and von Zastrow, 2008). GPCRs are differently susceptible to desensitization (Bristow *et al.*, 1986; Michel *et al.*, 1990; Summers *et al.*, 1997; Broadley, 1999). In addition, desensitization can markedly depend on the localization of a given receptor. β_2ARs on human bronchial smooth muscles are more resistant to desensitization than β_2ARs on human lymphocytes (Johnson, 2006).

1.3 Future directions in the investigation of GPCRs

Early descriptions of GPCRs as “on-off” switches resulting in the modulation of a single uniform signaling event are obsolete (Kenakin, 2001). At present, GPCRs are rather considered as “microprocessors”, navigating diverse intracellular signaling events (Kenakin, 2009). The behavior of a single GPCR is influenced by a complex interplay of numerous factors, which render the interpretation of pharmacological data more and more complex. Some of these factors, which are gaining interest in the GPCR research field, are discussed in the following.

1.3.1 G protein-independent signaling *via* β-arrestin

Besides the aforementioned classical G protein-dependent signal transduction, there is rapidly growing evidence of G protein-independent signaling of GPCRs (Luttrell, 2008). One example of the latter is signaling *via* β-arrestins 1 and 2, which have originally been regarded as regulators of GPCR desensitization (Lefkowitz and Shenoy, 2005). β-arrestins 1 and 2 are expressed in virtually all tissues, indicating their high relevance for regulation of GPCR signaling in general (Lefkowitz and Whalen, 2004). β-Arrestins as a multifunctional scaffold or as adapter proteins possess the ability to signal through numerous

pathways mediated e.g. by mitogen-activated protein kinases (MAPKs), phosphatidylinositol-3-kinase (PI3K), non-receptor tyrosine kinase SRC and nuclear factor-κB. Consequently, β-arrestins 1 and 2 are co-navigators of crucial cellular processes such as gene transcription, cell growth and differentiation (Rajagopal *et al.*, 2010). In contrast to rapid and transient G protein-mediated responses, β-arrestin-mediated effects are considered low-level and long-lasting (Kenakin and Miller, 2010). β-arrestin-signaling, triggered by numerous receptors, appears to be pro-survival, cytoprotective and anti-apoptotic (Violin and Lefkowitz, 2007). For example, β-arrestin-mediated signaling after activation of the β_1AR was reported to have cardioprotective effects in mice (Noma *et al.*, 2007). Therefore, it is not surprising, that this novel research area is gaining interest not only because some previously unexplained GPCR effects can be re-evaluated, but also because of its high clinical relevance.

Several methods have already been applied in the investigation of GPCR-β-arrestin interactions. Redistribution of fluorescently-labeled β-arrestin to activated GPCR can be visualized by fluorescence microscopy (Hudson *et al.*, 2006). Proximity assays monitor the distance between β-arrestin and GPCR after ligand binding by means of enzyme fragment complementation, bioluminescence (BRET) or Förster resonance energy transfer (FRET) (Rajagopal *et al.*, 2010). Whereas the aforementioned approaches monitor only receptor-β-arrestin interaction, measurement of phosphorylation of extracellular regulated kinases 1/2 (ERK1/2) enables the assessment of β-arrestin-mediated functional consequence of GPCR activation as ERK1/2 are regulated by β-arrestins (Violin and Lefkowitz, 2007). A drawback of the latter method is the fact that ERK1/2 can also be phosphorylated *via* G protein-dependent signals (Galandrin *et al.*, 2008; Evans *et al.*, 2010). In addition, genetically modified systems such as transgenic animals (knockout of β-arrestin or GRKs) or knockdown of β-arrestin with small interfering RNA technology enable the investigation of β-arrestin-signaling pathways *in vivo* (Noma *et al.*, 2007).

1.3.2 Functional selectivity

The complexity of GPCR signaling is further increased by the ability of ligands to differentially activate distinct signaling pathways by stabilizing ligand-specific conformation of a given receptor. In literature this observation is frequently termed functional selectivity (Galandrin *et al.*, 2007). As a given GPCR can promiscuously couple to different G proteins ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$) and additionally trigger downstream responses *via* β-arrestin, structurally different ligands are able to individually activate and/or inactivate multiple signaling pathways simultaneously. For example, the β-adrenoceptor antagonist carvedilol, successfully used in the therapy of heart failure, has β_2AR-mediated inverse agonistic properties regarding the G_s-dependent activation of ACs, and is an agonist for β-arrestin-dependent ERK1/2 phosphorylation in HEK 293 cells (Wisler *et al.*, 2007). Whereas (*R*,*R*)-

fenoterol was reported only to couple to G_s proteins in rat cardiomyocytes, (*S*,*R*)-fenoterol couples dually to G_s and G_i proteins after activation of the β_2AR in the same test system (Seifert and Dove, 2009; Woo *et al.*, 2009). Moreover, ligand-directed signaling was reported for the H_2R and the H_4R in recombinant test systems (Appl *et al.*, 2011; Rosethorne and Charlton, 2011). Very recently, Khasai and coworkers provided additional evidence for predominantly ligand-specific, and not agonist/inverse agonist-specific, conformations of the β_2AR using quantitative mass spectrometry, supporting the concept of functional selectivity (Kahsai *et al.*, 2011).

There is no doubt that functionally selective ligands have therapeutic relevance in some cases (Kenakin and Miller, 2010). Selective activation of G_s signaling *via* β_2AR is discussed to be beneficial in the treatment of congestive heart failure, whereas G_i-signaling could have detrimental effects (Woo *et al.*, 2009). However, the vast majority of data on functional selectivity were obtained from transiently transfected cells (Rajagopal *et al.*, 2010). As functional selectivity also depends on the cell type used, there is a growing interest to expand investigations on animal models and functionally intact human primary cells as physiologically more relevant test systems. New technologies have been developed, where dynamic mass redistribution in label-free cells after GPCR-activation is monitored by measuring changes of electrical impedance or by using optical biosensors. In both cases, real-time data provide unique kinetic patterns that can be used to recognize specific signaling pathways like G_s-, G_i- or G_q-mediated responses (Kenakin, 2009).

1.3.3 Formation of homo- and heteromers

Providing even more versatile and fine-tuned effects, GPCRs can modulate G protein-dependent and -independent responses not only as monomeric units, but also as homomers (two or more identical GPCRs in complex) and heteromers (two or more distinct GPCRs in complex) (Smith and Milligan, 2010). Binding of a particular ligand to homo- and heteromers can trigger responses distinct from those caused by monomeric GPCRs, because in higher-order GPCR-complexes the conformation of monomeric units is altered. For instance, co-stimulation of classically G_s-coupled dopamine D_1 receptor and G_i-coupled dopamine D_2 receptors, which were reported to form D_1-D_2 heterodimers, led to the stimulation of a solely G_q-mediated Ca^{2+} increase *in vitro* (Lee *et al.*, 2004). Moreover, probably the most prominent evidence for the formation of functional GPCR homodimers *in vivo* is a recent report of Rivero-Müller and colleagues (2010). Crossbreeding of mice with a binding-deficient version of luteinizing hormone (LH) receptor and mice with a signaling-deficient LH receptor rescued the wild-type phenotype with normal LH signaling.

Methods for the investigation of homo- and heterodimers comprise classical approaches like radioligand binding studies and co-immunoprecipitation as well as optical

methods like BRET, FRET and functional reconstitution of impaired monomers through dimerisation (Smith and Milligan, 2010). In addition, tracking of receptor dimerization by application of bivalent ligands is becoming increasingly popular (Shonberg *et al.*, 2011). Bivalent ligands are compounds with two pharmacophoric moieties, joined by a linker, becoming able to simultaneously target two distinct binding sites on a monomer or dimer (Smith and Milligan, 2010). However, despite occasionally extremely high potencies in recombinant test systems and animal models, unfavorable physicochemical properties of bivalent ligands are probably the main hurdle on their way to become clinically relevant candidates (Birnkammer, 2011; Shonberg *et al.*, 2011). Characterization of highly potent H_2R bivalent agonists in human primary cells was one of the subjects of this work (cf. chapters 2 and 4).

Keeping in mind G protein-dependent and -independent signaling, functional selectivity of particular ligands, formation of receptor homo- and heteromers as well as additional factors not discussed above (receptor reserve and compartmentation, susceptibility to desensitization), GPCRs can indeed be considered as “microprocessors” of information. A plethora of such signaling events can be mediated *via* a given GPCR as shown with a theoretical example in Fig. 1.2. Nevertheless, the development of compounds that preferentially stabilize a certain GPCR conformation that triggers event(s) of clinical benefit on the one hand while blocking adverse effect(s) on the other hand, will be a future challenge. Thus, for the characterization of such compounds diverse complementary predictive test systems are required (Kenakin, 2009). This issue has especially been considered in this work (cf. chapters 2, 3 and 4).

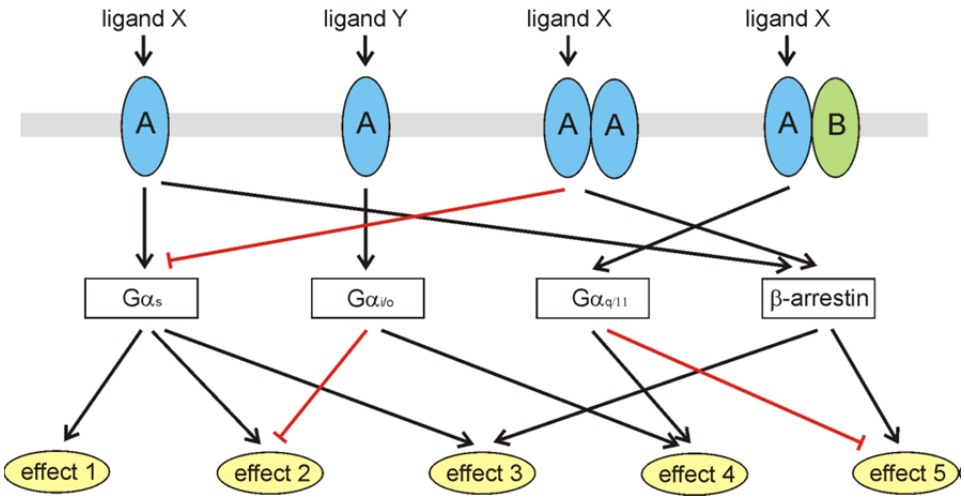


Fig. 1.2 Theoretical example for the complexity of GPCR-mediated signaling. After binding of the ligand X, the receptor A can signal differently as monomer, as homodimer and as heterodimer with receptor B. Ligands X and Y are able to modulate distinct downstream signaling events after binding to the same receptor, receptor A. Black lines stand for stimulating effect and red lines for inhibitory ones.

1.3.4 Extracellular regions of GPCRs

Extracellular regions of GPCRs, the N-terminus and the three extracellular loops, have been regarded as inert peptide linkers, enabling the correct positioning of functionally important transmembrane regions (Peeters *et al.*, 2011). In 2000, the first crystal structure of a class A GPCR, bovine rhodopsin, was resolved (Palczewski *et al.*, 2000). This structure enabled first insights into the three-dimensional architecture of a representative GPCR, where the second extracellular loop is positioned deeply in the binding cavity and is in direct contact with the bound ligand. Based on this observation, studies addressing the importance of extracellular regions of aminergic GPCRs are intensified and provided evidence for their involvement in ligand recognition, receptor activation, subtype and species selectivity as well as allosteric modulation (Shi and Javitch, 2002; Peeters *et al.*, 2011). Extracellular domains are the most variable part of GPCRs besides the C-terminus (Peeters *et al.*, 2011). Therefore, compounds targeting these regions could function as allosteric modulators with e.g. beneficial subtype selectivity (Bokoch *et al.*, 2010).

Valuable information on the location of amino acids in the extracellular area have been provided from resolved crystal structures of the human β_2-adrenergic receptor (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Rosenbaum *et al.*, 2007; Rasmussen *et al.*, 2011a; Rasmussen *et al.*, 2011b; Rosenbaum *et al.*, 2011), the turkey β_1-adrenergic receptor (Warne *et al.*, 2008), the human histamine H_1 receptor (Shimamura *et al.*, 2011) and others. Nevertheless, extracellular regions as highly flexible parts of receptors are often incompletely resolved in crystal structures, e.g. in the crystal structure of the human adenosine A_{2A} receptor (Jaakola *et al.*, 2008). Moreover, a limitation of crystal structures is that they only provide a snapshot-conformation of a given GPCR, therefore there is a lack of information about dynamics. By contrast, recent advances in nuclear magnetic resonance spectroscopy provide also information about the dynamic behavior of extracellular regions (Ahuja *et al.*, 2009; Tikhonova and Costanzi, 2009; Bokoch *et al.*, 2010). Most frequently classical mutagenesis studies, sometimes in combination with molecular dynamic simulation, are applied in the investigation of extracellular domains, an item also addressed in the present work (cf. chapters 2, 4 and 5).

1.4 Species-specificity of GPCRs

Mostly, GPCRs are considered as targets for compounds that could bring therapeutic benefit in humans. An investigation of drug candidates in translational animal models is indispensable before such compounds enter clinical trials. However, affinities of compounds at a given GPCR and the resulting biological effects can substantially differ from species to species. Species-dependent pharmacological properties of certain compounds

have been reported for the angiotensin AT_2 receptor (Feng *et al.*, 2005), the cannabinoid CB_2 receptor (Mukherjee *et al.*, 2004), the melanocortin 5 receptor (Huang *et al.*, 2000), the histamine H_3 receptor (Hancock, 2006; Schnell *et al.*, 2010) and the gondotropin-releasing hormone receptor (Reinhart *et al.*, 2004), to name only few. High amino acid homology between species in no guarantee for an identical pharmacological profile of a compound as in some cases the mutation of a single amino acid has a dramatic impact as shown for example for the 5-HT_{1B} receptor (Oksenberg *et al.*, 1992).

Therefore, tissue and species-specificity of GPCRs should be addressed early in the drug discovery process in order to avoid ineffectiveness of drugs in clinical trials and to reduce side effects. Species-specific behavior can already be investigated in a very early stage of drug development e.g. according to a high-throughput screening approach (Swanson and Beasley, 2010). Mutagenesis studies are frequently applied in order to identify single amino acids or regions, responsible for a divergence in affinity and functionality of test compounds in different species (cf. chapters 4 and 5) (Oksenberg *et al.*, 1992; Lim *et al.*, 2008; Strasser *et al.*, 2008). Moreover, computer-aided homology models and molecular dynamic simulations can provide useful information on species-specific interactions of compounds with GPCRs (cf. chapter 5) (Schnell *et al.*, 2010). The generation of transgenic animals, in which the rodent GPCR is replaced by the human GPCR (knockin animals, e.g. humanized mouse) is another strategy to overcome species-specific problems (Gladue *et al.*, 2006).

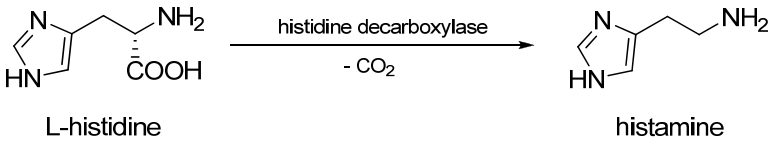
1.5 The histaminergic system

1.5.1 Histamine as endogenous ligand

HA (2-(1*H*-imidazol-4-yl)ethanamine) is a local mediator, immunomodulator and neurotransmitter targeting the histaminergic system. First biological effects of HA like vasodilatation and smooth muscle contraction were reported more than one hundred years ago (Dale and Laidlaw, 1910). Throughout the last century our understanding of histamine and its (patho)physiological role has increased enormously and resulted in the discovery of blockbuster drugs in the therapy of, e.g., allergic reactions and peptic ulcer. Still, because of their involvement in disorders of the central nervous system (CNS) and immune system, HA-mediated effects are very active areas of research.

The main sources of histamine are mast cells and basophil granulocytes (Bäumer and Rossbach, 2010). Moreover, histamine is also stored in enterochromaffin-like cells of the stomach (Prinz *et al.*, 1999), blood platelets (Saxena *et al.*, 1989), in neurons (Haas *et al.*, 2008), and it is present in many other tissues (Zimmermann *et al.*, 2011). HA is synthesized in the body from the amino acid L-histidine through decarboxylation (Fig. 1.3). This reaction

is catalyzed by histidine decarboxylase (Haas *et al.*, 2008). Normally, HA is then translocated from the cytosol into secretory vesicles by the vesicular monoamine transporter $VMAT_2$ (Dimaline and Struthers, 1996). In addition, in various cells like macrophages, dendritic cells, neutrophils and T cells HA is synthesized *de novo* (Thurmond *et al.*, 2008; Smuda and Bryce, 2011). After release in response to immunological and non-immunological stimuli, HA is degraded by two catabolic pathways. The first pathway involves methylation of HA by histamine *N*-methyltransferase and the second pathway involves oxidative deamination by diamine oxidase (Ogasawara *et al.*, 2006).



L-histidine histamine

Fig. 1.3 Biosynthesis of histamine.

Histamine mediates its pleiotropic effects by targeting four histamine receptor subtypes, referred to as histamine H_1 (H_1R), H_2 (H_2R), H_3 (H_3R) and H_4 receptor (H_4R). The activated H_1R couples to $G_{q/11}$ proteins and promotes typical effects of allergic reactions like increased vascular permeability (Hill *et al.*, 1997). The H_3R signals *via* $G_{i/o}$ proteins and is primarily involved in CNS functions like cognition, learning, memory and emotion (Haas *et al.*, 2008). Because of being in focus of the present work, the H_2R and H_4R are more extensively discussed in the next two sections.

1.5.2 The histamine H_2 receptor

The existence of the H_2R subtype was confirmed in 1972 by Black and coworkers (Black *et al.*, 1972). Their observation that, in contrast to the H_1R antagonist mepyramine, burimamide inhibited the HA-induced gastric acid secretion in anaesthetized rats and the HA-stimulated increase in heart rate at the isolated guinea pig right atrium led to the classification of burimamide as the first H_2R antagonist. Almost twenty years later, molecular cloning of the H_2R from canine and human gastric parietal cells by Gantz and coworkers triggered extensive characterization of the H_2R also in recombinant test systems (Gantz *et al.*, 1991a; Gantz *et al.*, 1991b).

The gene encoding for the human H_2R (hH_2R) is located on the chromosome gene locus 5q35.2 (Traiffort *et al.*, 1995). The overall amino acid sequence homology between the hH_2R and the H_2R from other species like canine (dog), guinea pig, mouse and rat is

relatively high (> 80 %) (Gantz *et al.*, 1991a; Gantz *et al.*, 1991b; Ruat *et al.*, 1991; Traiffort *et al.*, 1995; Kobayashi *et al.*, 1996). Two isoforms of the hH_2R are listed in the database of the National Center for Biotechnology Information, isoform 1 with 397 amino acids and isoform 2 with 359 amino acids (cf. chapter 3) (Strausberg *et al.*, 2002). Several single nucleotide polymorphisms (SNP) in the promoter and encoding region of the hH_2R gene have been identified in different populations, but reports on the pathological relevance in schizophrenia are controversial (Orange *et al.*, 1996; Ito *et al.*, 2000; Mancama *et al.*, 2002). Moreover, formation of H_2R dimers and higher-degree complexes was reported in recombinant test systems (Fukushima *et al.*, 1997).

The H_2R predominantly couples to G_s proteins, resulting in an activation of ACs and the subsequent formation of cAMP (Hill *et al.*, 1997) (Fig. 1.4). The second messenger cAMP activates PKA, which, in turn, leads to modulation of numerous downstream signaling pathways like activation of transcription factor termed cAMP response-element binding (CREB) protein resulting in altered gene transcription (Bakker and Leurs, 2005). In addition to G_s coupling, the coupling to $G_{q/11}$ proteins was observed for the H_2R in some but not all cell systems (Seifert *et al.*, 1992; Kühn *et al.*, 1996; Wellner-Kienitz *et al.*, 2003). The consequence of $G_{q/11}$-coupling is an increase in intracellular Ca^{2+} concentration mediated by the PLC. Constitutive activity of the H_2R was observed in some recombinant test systems (Smit *et al.*, 1996; Seifert and Wenzel-Seifert, 2002; Preuss *et al.*, 2007).

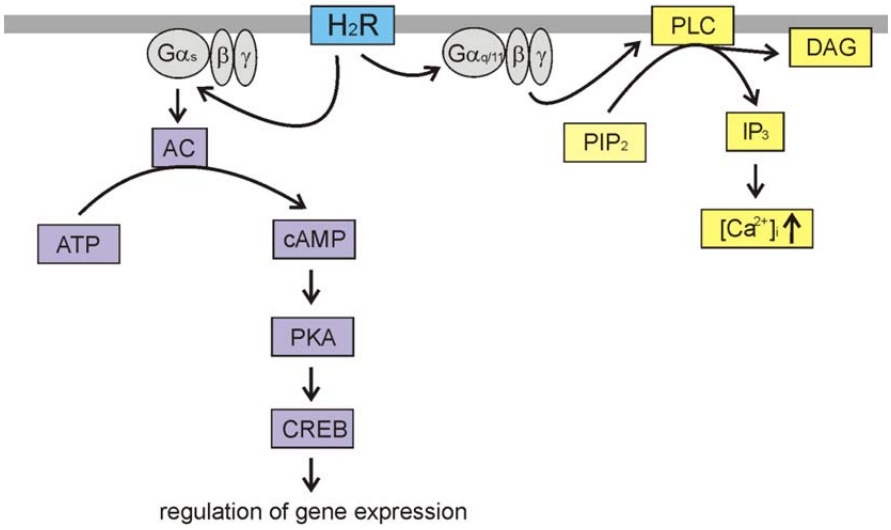


Fig. 1.4 Signal transduction pathways activated by the H_2R stimulation.

The H_2R is expressed in numerous tissues. In gastric parietal cells, the H_2R is responsible for the HA-mediated stimulation of gastric acid secretion (Black *et al.*, 1972). Activation of H_2Rs in atrial and ventricular tissues results in positive chronotropic and inotropic effects (Levi *et al.*, 1982). Moreover, H_2R-mediated smooth muscle relaxation in

airways, blood vessels and the uterus was reported (Black *et al.*, 1972; Eyre and Chand, 1982; Levi *et al.*, 1982). Activated H_2Rs on neuronal membranes in the CNS normally potentiate excitation, e.g. by inhibiting long-lasting hyperpolarisation (Haas and Panula, 2003). The H_2R is also expressed on numerous cells of the immune system like monocytes, dendritic cells, Th1/2 cells, mast cells, eosinophils and neutrophils (Bäumer and Rossbach, 2010). H_2Rs are negative regulators of the Th1- and Th2-mediated immune response (Jutel *et al.*, 2001; Kunzmann *et al.*, 2003) and are involved in the modulation of cytokine production (Akdis and Simons, 2006). Cimetidine was reported to have anti-tumor activity by inhibiting the H_2R-induced suppression of the immune system (Lefranc *et al.*, 2006). Finally, stimulation of H_2Rs on promyeloid leukemic cells induces their differentiation into mature granulocytes (Seifert *et al.*, 1992; Klinker *et al.*, 1996), therefore, H_2R agonists could be beneficial in the therapy of acute myeloid leukemia.

The selective H_2R antagonists cimetidine, ranitidine, famotidine, nizatidine and roxatidine have been successfully used in the treatment of gastric and duodenal symptoms (ulcers), but are nowadays mostly replaced by more effective proton pump inhibitors (Parsons and Ganellin, 2006).

1.5.3 The histamine H_4 receptor

Although the existence of an additional histamine receptor subtype besides the H_1R, H_2R and H_3R was predicted long ago (Raible *et al.*, 1994), the H_4R was discovered not until the year 2000 (Nakamura *et al.*, 2000; Oda *et al.*, 2000; Liu *et al.*, 2001a; Morse *et al.*, 2001; Nguyen *et al.*, 2001; Zhu *et al.*, 2001). Extensive characterization of the H_4R in the past decade contributed to a better understanding of some symptoms of allergic reactions and asthma that could not be explained by the action of HA at the other three histamine receptors subtypes. Nevertheless, numerous questions about the (patho)physiology of the H_4R remain to be answered.

The gene for the human H_4R (hH_4R) is located on the chromosome gene locus 18q11.2 of the human genome (Haas *et al.*, 2008). Full-length hH_4R consists of 390 amino acids. In contrast to other histamine receptor subtypes, the amino acid sequence homology between different species is quite low for the H_4R. For example, hH_4R shares only between 65 and 71 % sequence homology with guinea pig, mouse, rat and canine H_4Rs (Liu *et al.*, 2001b; Jiang *et al.*, 2008). Two splice variants of the full-length hH_4R were detected in eosinophils and mast cells, H_4R_{67} and H_4R_{302}. Both splice variants are non-functional as monomers but can negatively regulate the full-length hH_4R presumably by forming hetero-oligomers (van Rijn *et al.*, 2008). Various SNPs were detected in the encoding and intron regions of the hH_4R gene and some of them are associated with atopic dermatitis (Leurs *et*

al., 2009; Yu *et al.*, 2010). The formation of homodimers of the recombinant and endogenously expressed hH_4R was observed by van Rijn and coworkers (2006).

The H_4R couples to members of the PTX-sensitive $G_{i/o}$ proteins (Fig. 1.5). Thus, activation of the H_4R reduces cAMP formation and further downstream events like CREB-mediated gene transcription (Leurs *et al.*, 2009). In addition, the H_4R can activate the MAPK pathway *via* PTX-sensitive mechanisms (Morse *et al.*, 2001). Furthermore, activation of H_4R in mast cells and eosinophils leads to a mobilization of intracellular $[Ca^{2+}]_i$ (Buckland *et al.*, 2003; Hofstra *et al.*, 2003). An increase in $[Ca^{2+}]_i$ is sensitive to PTX and PLC inhibitors, indicating that PLC is activated by the dissociated $G\beta\gamma$ subunit after H_4R activation (de Esch *et al.*, 2005). Recently, signaling of the H_4R, presumably *via* a G protein-independent β-arrestin pathway, resulting in a phosphorylation of ERK 1/2 in U2OS cells has been reported (Rosethorne and Charlton, 2011; Seifert *et al.*, 2011). The H_4R exerts high levels of constitutive activity (Morse *et al.*, 2001; Schneider *et al.*, 2009).

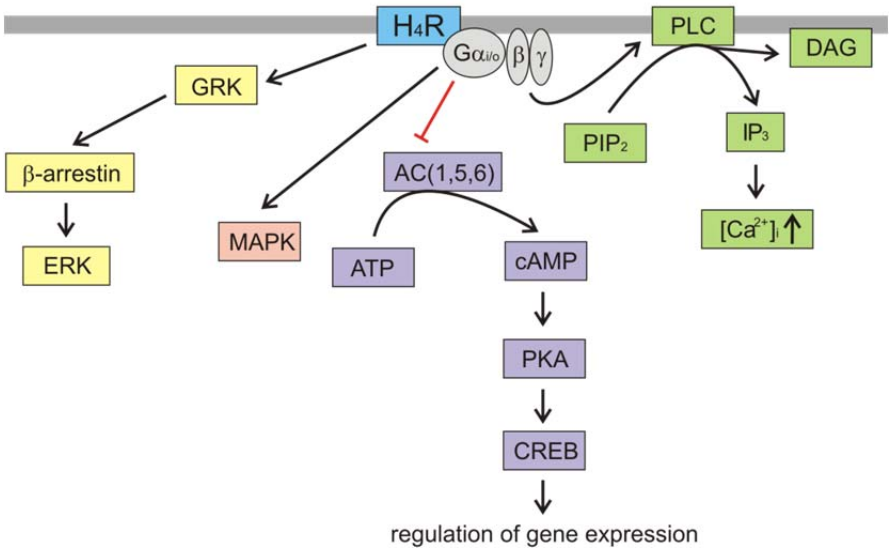


Fig. 1.5 Signal transduction pathways activated by the H_4R stimulation. In general, among ten AC isoforms, only isoforms 1, 5 and 6 can be inhibited by $G\alpha_{i/o}$ proteins (Tang and Hurley, 1998; Sadana and Dessauer, 2009).

The H_4R is preferentially expressed in cells of the immune system, in particular in mast cells, eosinophils, T cells, dendritic cells, basophils and monocytes, and many reports implicate its role in the modulation of immune and inflammatory responses (Thurmond *et al.*, 2008; Bäumer and Rossbach, 2010). The H_4R induces chemotaxis of mast cells, eosinophils, dendritic cells and T cells *in vitro* (Hofstra *et al.*, 2003; Ling *et al.*, 2004; Gutzmer *et al.*, 2005; Morgan *et al.*, 2007). Furthermore, HA acting through the H_4R stimulates upregulation of adhesion molecules, actin polymerization and shape changes of eosinophils (Buckland *et al.*, 2003; Ling *et al.*, 2004). Moreover, a role of the H_4R is considered in the modulation of

various cytokine responses, leading e.g. to the Th2 T cell polarization relevant in the pathophysiology of asthma (Thurmond *et al.*, 2008). A beneficial effect of H_4R antagonism was confirmed *in vivo*, where H_4R-deficiet mice and mice treated with H_4R antagonist JNJ7777120 (1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine) showed reduction of allergic lung inflammation accompanied by decreased Th2 response (Dunford *et al.*, 2006). In addition, H_4R antagonism proved to be effective by inhibiting longer-term Th2 cytokine-driven pathologies like lung remodeling in a sub-chronic asthma model in mice (Cowden *et al.*, 2010). Furthermore, beneficial anti-inflammatory effects of H_4R antagonism have been observed in acute pruritus models in mice (Dunford *et al.*, 2007; Rossbach *et al.*, 2011) and an acute colitis model in rat (Varga *et al.*, 2005). Moreover, H_4R antagonists of 2-aminopyrimidine class, structurally different from JNJ7777120, had pain-reducing effect in rat (Cowart *et al.*, 2008; Liu *et al.*, 2008) and reduced inflammation in zymosan-induced peritonitis in mice (Strakhova *et al.*, 2009). Expression of the H_4R was also reported in synovial cells of patients with rheumatoid arthritis, suggesting an involvement of the receptor in the pathophysiology of this disease (Ohki *et al.*, 2007). The use of H_1R antagonists in combination with H_4R antagonist JNJ7777120 showed a synergistic inhibitory effect on the HA-induced scratching in mice (Dunford *et al.*, 2007) and eosinophil infiltration into bronchoalveolar lavage fluid in an acute murine asthma model (Deml *et al.*, 2009), indicating that combined H_1R/H_4R antagonism may also entail benefits compared to monotherapy with classical H_1R antagonists.

Although the majority of reports suggests pro-inflammatory effects as a consequence of the H_4R activation, some recent reports are not in accordance with this dogma. JNJ7777120, used as a selective H_4R antagonist in numerous animal studies, has shown inverse agonistic activity at the hH_4R and partial agonistic activity at rat, mouse and dog H_4Rs in steady-state GTPase assay using Sf9 insect cell membranes (Schneider *et al.*, 2010; Schnell *et al.*, 2011). Moreover, the same ligand behaved as an agonist in a β-arrestin recruitment assay (Rosethorne and Charlton, 2011). In addition, a possible anti-inflammatory role of the H_4R in a murine asthma model has been discussed recently (Neumann *et al.*, 2010). And last but not least, very recently, no beneficial effect of H_4R antagonists was observed in canine model of acute atopic dermatitis (Bäumer *et al.*, 2011). Therefore, new selective H_4R ligands and additional *in vitro* and *in vivo* studies are needed to provide conclusive evidence about beneficial effects of the H_4R-modulation in inflammatory and immunological disorders like bronchial asthma, chronic pruritus and rheumatoid arthritis (Zampeli and Tiligada, 2009).

Eleven years after its discovery, ligands targeting the H_4R subtype are not yet on the market. The H_4R antagonist UR-63325 (undisclosed structure, Palau Pharma, Spain) is

currently in the phase II clinical trial, where its effectiveness in the therapy of asthma and allergic rhinitis is investigated (Leurs *et al.*, 2011).

1.6 The adrenergic system

1.6.1 The endogenous ligands adrenaline and noradrenaline

The adrenergic system is responsible for a variety of physiological responses in the central and peripheral nervous system also named “fight-or-flight” responses. The endogenous ligands of this system are catecholamines adrenaline (ADR, 4-[(1*R*)-1-hydroxy-2-(methylamino)ethyl]benzene-1,2-diol), commonly referred to as epinephrine, and noradrenaline (NA, 4-[(1*R*)-2-amino-1-hydroxyethyl]benzene-1,2-diol), also referred to as norepinephrine. The biosynthesis of NA and ADR is shown in Fig. 1.6. The synthesis of NA takes place in noradrenergic neurons, whereas ADR is finally synthesized mainly in the medulla of the adrenal gland (Hein, 2004). After release from postganglionic sympathetic nerves and adrenal medulla, the action of ADR and NA is terminated by reuptake into nerve terminals. By analogy with other catecholamines, ADR and NA are metabolized by two enzymes, monoamine oxidase and catechol-O-methyl transferase (Kopin, 1994; Oeltmann *et al.*, 2004).

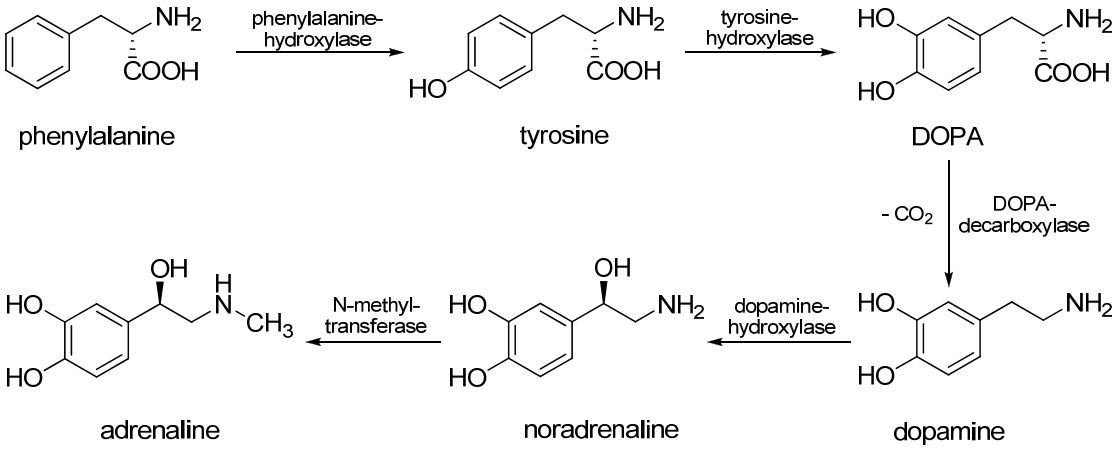


Fig. 1.6 Biosynthesis of noradrenaline and adrenaline.

The hormone ADR and the neurotransmitter NA trigger diverse effects in the human body by targeting the α_1-adrenergic receptors (α_1AR), the α_2-adrenergic receptors (α_2AR) and the β-adrenergic receptors. The latter are subdivided into the β_1AR, β_2AR and β_3AR subtypes (http://www.iuphar-db.org/index.jsp). α_1ARs couple to $G_{q/11}$ proteins and play an important role in the control of smooth muscle tonus whereas α_2ARs, $G_{i/o}$-coupled receptors, mainly act as negative regulators of neurotransmitter release (Minneman and Esbenshade, 1994;

Philipp and Hein, 2004). β_1ARs and β_3ARs classically couple to G_s proteins and their activation results in increased cardiac contractile force and heart rate as well as increased lipolysis, respectively (Hein, 2004).

1.6.2 The β_2-adrenergic receptor

The existence of the β_2AR subtype was suggested by Lands and colleagues on the basis of the observation that certain agonists and antagonists could be used to pharmacologically discriminate between βAR-mediated effects among tissues like cardiac muscle and bronchial smooth muscle (Lands *et al.*, 1967). The first recombinant β_2AR, cloned from hamster genomic library, was available to the research community nearly twenty years later (Dixon *et al.*, 1986). At present, the β_2AR is probably the best characterized GPCR.

The encoding region of the human β_2AR (hβ_2AR) gene is located on chromosome 5q31 (Liggett, 1997). The hβ_2AR consists of 413 amino acids and shares high sequence homology with β_2ARs of other species like hamster (87 %), guinea pig (88 %), dog (90 %) and cattle (88 %) (Kobilka *et al.*, 1987; Huang *et al.*, 1997; Einspanier *et al.*, 1999; Oostendorp *et al.*, 2002). More than 80 SNPs of the hβ_2AR were identified so far (Gnadt, 2011). Among them, three SNPs that alter the amino acid sequence of the hβ_2AR were suggested to have significant clinical relevance in the therapy of asthma (Liggett, 1997; Johnson, 2006). The SNP corresponding to amino acid position 16 (arginine or glycine) has influence on receptor down-regulation after exposure to an agonist. Moreover, glutamate at position 27 (instead of glutamine) seems to protect receptor against down-regulation. Another SNP is located in the TM IV at amino acid position 164 (threonine or isoleucine) and alters agonist-binding properties of the β_2AR, also resulting in altered coupling to further downstream signaling pathways. The β_2AR forms homodimers as well as heterodimers with the β_1AR, β_3AR, angiotensin type 1 receptor and others (Smith and Milligan, 2010). Like the H_2R and H_4R, the β_2AR is constitutively active (Seifert and Wenzel-Seifert, 2002).

The β_2AR classically couples to G_s proteins, leading to AC activation and cAMP production and consequently to PKA activation (Johnson, 2006). Further downstream signaling events are e.g activation of p38 MAPK (Zheng *et al.*, 2000) and relaxation of smooth muscle, where PKA is responsible for the phosphorylation of regulatory proteins controlling the availability of Ca^{2+} and myosin light-chain kinase activity (Anderson, 2006). In addition to G_s coupling, the β_2AR interacts with G_i proteins, resulting in an activation of ERK1/2 and p38 MAPK *via* Gβγ protein (Evans *et al.*, 2010). Phosphorylation of the β_2AR by PKA was reported to switch the coupling of the receptor from G_s to G_i proteins (Daaka *et al.*, 1997). However, PKA-dependent G_i-coupling of the β_2AR remains controversial (Friedman *et al.*, 2002; Seifert and Dove, 2009). Moreover, the recruitment of β-arrestin, which is primarily

responsible for receptor desensitization, to the β_2AR leads to the ERK1/2 and p38 MAPK activation in a G protein-independent manner (Azzi *et al.*, 2003; Gong *et al.*, 2008). A simplified depiction of complex promiscuous signaling of the β_2AR, described extensively by Evans *et al.* (2010), is given in Fig. 1.7.

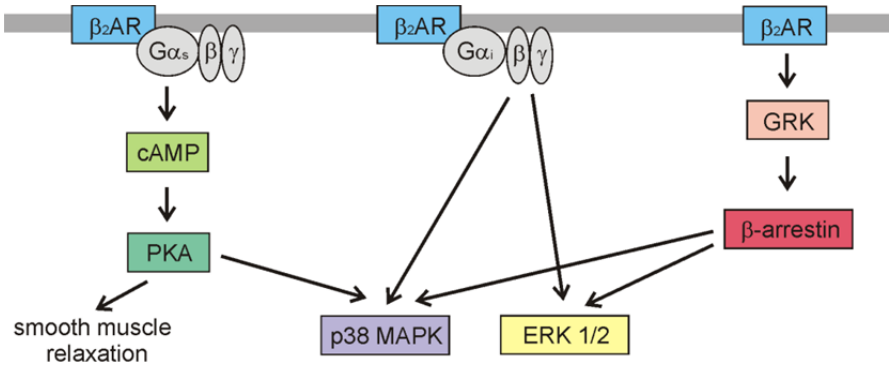


Fig. 1.7 Simplified depiction of cross-talk of signaling pathways upon β_2AR activation. Adapted from Evans *et al.* (2010).

The β_2AR is widely distributed in the lung. The highest density of the β_2AR was found in smooth muscle cells of the respiratory tract (30,000 - 40,000 receptors per cell) and it is also expressed in pulmonary epithelial and endothelial cells and mast cells (Johnson, 2006). Activated β_2ARs in airways elicit bronchodilatation and inhibit liberation of inflammatory mediators like HA and cytokine production after an allergen challenge (O'Connor *et al.*, 1992; Anderson, 2006). In addition, β_2ARs, localized in myocardium and atrial-ventricular conducting system, regulate heart force and rate (Anderson, 2006). Relaxation of uterine, vascular and gastrointestinal smooth muscles can be achieved *via* β_2ARs (Tanaka *et al.*, 2005). Presynaptic localisation of β_2ARs on the noradrenergic axons is responsible for stimulation of neurotransmitter release (Ariens and Simonis, 1983). And last but not least, the β_2AR is also expressed on inflammatory cells like mast cells, monocytes, T cells, eosinophils and neutrophils. β_2AR agonists, mainly used as bronchodilatators, additionally inhibit activation of inflammatory cells, their recruitment to bronchoalveolar lavage and release of inflammatory mediators (Johnson, 2002).

β_2AR agonists are used in therapy of asthma and to a lesser extent in chronic obstructive pulmonary disease. Short-acting β_2AR agonists (SABA) like salbutamol and fenoterol with rapid onset are used in acute asthma attacks whereas long-acting β_2AR agonists (LABA) like salmeterol, formoterol and indicaterol are used in long-term control of asthma symptoms (Sears and Lötvall, 2005; Gnadt, 2011). β_2AR agonists such as salbutamol are also used as myometrial relaxants to prevent premature labor (Gill *et al.*, 2006).

1.7 Short introduction to the test systems used in the thesis

1.7.1 Neutrophil granulocytes as a test system for characterizing H_2Rs and β_2ARs

Neutrophil granulocytes or polymorphonuclear neutrophils are white blood cells from the myeloid lineage (Galli *et al.*, 2011). As one of the key players of the innate immune response, neutrophils are able to phagocytose, kill and digest invading microorganisms (Segal, 2005). Neutrophil function is, among others, regulated by numerous GPCRs on the plasma membrane, e.g. the formyl peptide receptor, the platelet-activating factor receptor, the complement factor 5a receptor, prostaglandin receptors, the H_2R and the β_2AR (Omann *et al.*, 1987; Seifert and Schultz, 1991).

Generally, pro-inflammatory responses of neutrophil granulocytes are inhibited by cAMP-increasing agents (Moore and Willoughby, 1995; Flamand *et al.*, 2004). Therefore, H_2R- and β_2AR-mediated effects on neutrophil granulocytes are mainly of the anti-inflammatory type, because both receptors classically couple to G_s proteins (Gespach and Abita, 1982; Lad *et al.*, 1985; Bäumer and Rossbach, 2010). In the following, few examples of H_2R- and β_2AR-dependent effects are mentioned. HA inhibits N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)- and platelet-activating factor-induced leukotriene (LT) synthesis in human neutrophils *via* the H_2R (Flamand *et al.*, 2004). The H_2R negatively regulates neutrophil infiltration to the site of inflammation (Hirasawa *et al.*, 2002). The β_2AR agonist formoterol attenuates fMLP-induced synthesis of LTB_4 (Gravett *et al.*, 2010). In addition, β_2AR agonists suppress peroxidase release from neutrophil granules (Yasui *et al.*, 2006). Activation of both receptors leads also to the inhibition of NADPH oxidase that is crucial enzyme for initiation of the respiratory burst (Burde *et al.*, 1989; Betten *et al.*, 2003; Yasui *et al.*, 2006).

Collectively, the influence of the H_2R and the β_2AR on intracellular events in neutrophil granulocytes can be monitored by different downstream read-outs. Moreover, neutrophils are the most abundant white blood cells in humans and can be easy isolated from venous blood in sufficient quantities (Selvatici *et al.*, 2006). Hence, isolated human neutrophils have been used as a physiologically relevant test system for the characterization of ligands targeting the H_2R and the β_2AR in the present work (cf. chapters 3 and 4).

1.7.2 The Sf9 insect cell system

Sf9 insect cells are derived from *Spodoptera frugiperda* pupal ovarian tissue and are suitable for high-level expression of functional GPCRs and other proteins (Schneider and Seifert, 2010). For instance, the hβ_2AR was purified from Sf9 insect cells in sufficient quantities to allow its crystallization (Cherezov *et al.*, 2007). The GPCR of interest can be recombinantly expressed by infection of the Sf9 cells with genetically modified baculoviruses

(*Autographa californica*) that carry the inserted gene encoding the GPCR of interest. An additional advantage of Sf9 cells is their ability to perform most post-translational modifications of GPCR present in mammalian cells (Aloia *et al.*, 2009). Sf9 insect cells endogenously express G_q- and G_s-like proteins as well as one G_i isoform (Kühn *et al.*, 1996; Knight and Grigliatti, 2004). Mammalian GPCRs couple only poorly to insect G_q and G_s proteins (Houston *et al.*, 2002; Dolby *et al.*, 2004). G_i proteins from Sf9 cells normally do not couple to mammalian GPCRs as was also shown for the hH_4R (Schneider *et al.*, 2009; Schneider and Seifert, 2010). Therefore, limited coupling of expressed mammalian GPCRs with endogenous G proteins ensures favorably high signal-to-noise ratios.

In an early stage of the drug development process, recombinant test systems are useful tool for providing some valuable mechanistic data by keeping the maintenance requirements low (Aloia *et al.*, 2009; Kenakin, 2009). As such, the Sf9 cell system enables the reconstitution of the GPCR of interest in a defined environment that precludes cross-talk with other receptors. Moreover, controlled co-expression of the selected GPCR and G protein(s) as well as the use of GPCR-G protein fusion proteins enables the precise assessment of the G protein coupling profile (Lachance et al., 1999; Gazi et al., 2003). Expression of GPCRs in Sf9 cells enables both, binding studies (e.g. high-affinity agonist binding) and functional studies like cAMP accumulation, [^{35}S]GTPγS binding to the Gα protein, GTPase activity of the Gα protein, Ca^{2+} mobilization and IP_3 formation (Schneider and Seifert, 2010). Experiments can be carried out either with whole cells (Zhang *et al.*, 1999) or with broken-cell preparations (membranes) as in the present study (cf. chapters 4 and 5).

1.8 References

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Chapter 2

Scope and Objectives

As outlined in the General Introduction, in drug research recombinant test systems are frequently applied in the characterization of G protein-coupled receptors (GPCRs). However, the question arises, if such artificial systems in deed represent the (patho)physiological situation and if pharmacological profiles of compounds determined at recombinant receptors are predictive for their behavior on native GPCRs. Therefore, there is a tendency toward the use of native test systems, where GPCRs are characterized in a more or less intact environment. Human neutrophil granulocytes represent such a native test system, where endogenously expressed histamine H_2 receptors (H_2Rs) and β_2-adrenergic receptors (β_2ARs) mediate anti-inflammatory responses of potential therapeutic relevance (Johnson, 2002; Bäumer and Rossbach, 2010). The first aim of this thesis was the characterization of both receptors on these immune cells. Precisely, the role of these GPCRs in the accumulation of cyclic adenosine 3',5'-monophosphate and in the production of superoxide anions should be evaluated. Results should be compared with published data, obtained for the H_2R and the β_2AR in recombinant test systems, in order to elucidate test system peculiarites. Moreover, under the same experimental conditions the analysis of the H_2R and the β_2AR should reveal a potential divergence in their anti-inflammatory action on neutrophils.

New selective H_2R agonists are not only needed as valuable pharmacological tools, but could also become potential drug candidates, e.g. in the therapy of severe acute myeloid leukemia. Monovalent and bivalent N^G-acylated hetarylpropylguanidines, developed in our laboratory, turned out to be highly potent H_2R agonists (Kraus *et al.*, 2009; Birnkammer, 2011). However, these ligands are generally more potent at the guinea pig than at the human H_2R, whereas standard H_2R agonists such as amthamine and dimaprit are equipotent at both species orthologs. Although already addressed in a previous study (Birnkammer, 2011), molecular determinants for the species selective profile of acylguanidines are not completely understood. A second object of this work was to explore by mutagenesis studies, if there is a potential involvement of the extracellular N-terminus of the H_2R in species-divergence.

The aforementioned acylguanidines are amphiphilic cationic compounds and therefore prone to interactions with biological membranes and off-targets. Moreover, they have cytotoxic and hemolytic activity at higher concentrations and bind strongly to serum albumin (Birnkammer, 2011). Acylguanidines have been characterized at the human H_2R only on Sf9 cell membranes so far. Under these conditions unfavorable physicochemical properties do obviously not hamper the effectiveness of the agonists. However, their physicochemical nature could be problematic with respect to hH_2R-mediated responses in live cells. To evaluate the drug-likeness of representative monovalent and bivalent

acylguanidines, their agonistic activity was intended to be evaluated at endogenously expressed H_2R on human neutrophil granulocytes.

Frequently occurring species-specific characteristics of GPCRs are a major problem because an examination of drug candidates in translational animal models is inevitable. In particular, the behavior of the histamine H_4 receptor (H_4R) orthologs depends largely on the species used (Jiang *et al.*, 2008; Lim *et al.*, 2010; Schnell *et al.*, 2011). However, which regions or amino acids of the H_4R are responsible for this divergence between species is poorly investigated. Amino acid homology of the extracellular N-teminus and three extracellular loops between e.g. human and canine H_4R is very low, and it is conceivable, that these regions contribute to species differences. Therefore, an additional aim of this work was to elucidate the importance of extracellular regions for the large species differences between the human and the canine H_4R. In order to achieve this aim, human/canine H_4R chimeras with modified extracellular regions should be constructed and the influence on expression in Sf9 cells as well as on ligand binding and receptor activation should be examined.

2.1 References

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Chapter 3

Distinct pharmacological profile of the histamine H_2 receptor and the β_2-adrenergic receptor on human neutrophil granulocytes

3.1 Introduction

In humans neutrophil granulocytes represent 50 to 60 % of the total white blood cells and are involved in defense of the host organism against invading infectious agents such as bacteria, fungi, protozoa, viruses and tumor cells (Selvatici *et al.*, 2006). After phagocytosis of invading agents neutrophils are able to destruct them by releasing proteases and antimicrobial proteins and by respiratory burst (Bertram and Ley, 2011). One important player in the process of the so-called respiratory burst is an enzymatic complex, namely the NADPH oxidase, localized on the plasma and phagosome membrane of neutrophils and some other cells of the immune system (Seifert and Schultz, 1991). This enzyme catalyzes the univalent reduction of molecular oxygen (O_2) to the superoxide anion ($O_2^{\bullet-}$) with NADPH as the specific electron donor according to the following reaction: $2O_2 + NADPH \rightarrow 2O_2^{\bullet-} + NADP^+ + H^+$ (Morel *et al.*, 1991; Seifert and Schultz, 1991). H_2O_2 is generated by superoxide dismutase from $O_2^{\bullet-}$ and further metabolized to other reactive oxygen species and hypochloric acid (Bertram and Ley, 2011).

Activation of neutrophil granulocytes is triggered by numerous intercellular molecules like platelet activating factor, adenosine 5'-triphosphate (ATP) and bacterial formyl peptides (Burde *et al.*, 1989). Upon binding of N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) to the formyl peptide receptor (FPR), which is G_i-coupled (Gierschik *et al.*, 1989; Wenzel-Seifert *et al.*, 1999), $O_2^{\bullet-}$ production in neutrophils increases (Selvatici *et al.*, 2006) (Fig. 3.1). This happens *via* activation of phospholipase C (PLC) resulting in the cleavage of phosphatidylinositol-4,5-bisphosphate (PIP_2) yielding 1,2-diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). DAG activates protein kinase C (PKC) and IP_3 induces Ca^{2+}-mobilization from intracellular stores (Morel *et al.*, 1991). The activation of PKC is required for the phosphorylation of NADPH oxidase resulting in its activation (Bertram and Ley, 2011).

fMLP-stimulated $O_2^{\bullet-}$ production in neutrophil granulocytes and HL-60 cells is counteracted by some agents that increase the intracellular cyclic adenosine 3',5'-monophosphate (cAMP) concentration (Seifert and Schultz, 1991). These agents are, among others, prostaglandins, the unspecific inhibitor of phosphodiesterases, 3-isobutyl-1-methylxanthine (IBMX), the cell-permeable cAMP analog dibutyryl cAMP as well as agonists of the histamine H_2 receptor (H_2R) and the β_2-adrenergic receptor (β_2AR) (Wong and Freund, 1981; Burde *et al.*, 1989; Seifert and Schultz, 1991; Mitsuyama *et al.*, 1995; Mirza *et al.*, 2002). Furthermore, fMLP-stimulated $O_2^{\bullet-}$ formation is enhanced by the incubation of neutrophils with N-(2-{[(E)-3-(4-bromophenyl)prop-2-enyl]amino}ethyl)isoquinoline-5-sulfonamide (H-89), an inhibitor of cAMP-dependent protein kinase (PKA) (Mitsuyama *et al.*, 1995). It is not exactly clear, how cAMP increasing agents counteract the fMLP-stimulated $O_2^{\bullet-}$ production, but mechanisms involving the phosphorylation of FPRs, G_i proteins, PLC and p47 subunit of NADPH oxidase by PKA are discussed (Seifert and Schultz, 1991). A newer

study suggests the H_2R-induced inhibition of fMLP-stimulated $O_2^{\bullet-}$ production to be upstream of PKC (Betten *et al.*, 2003).

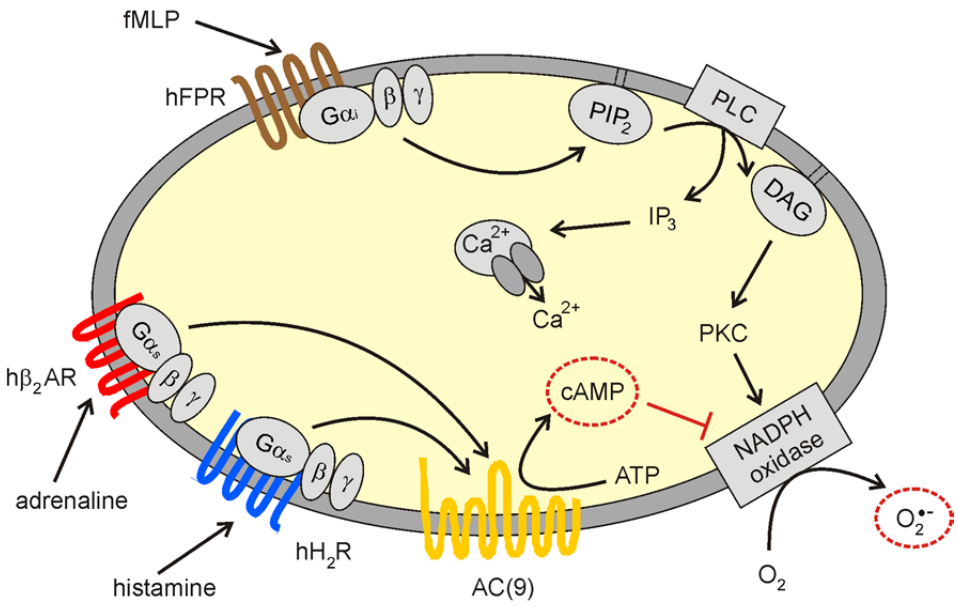

Fig. 3.1 Simplified depiction of the fMLP-induced $O_2^{\bullet-}$ production signaling pathway and the interference of the latter with the signaling pathways of the H_2R and β_2AR in human (h) neutrophil granulocytes. $O_2^{\bullet-}$ and cAMP accumulation (marked with red circles) were chosen as read outs for the characterization of the hH_2R and the $h\beta_2AR$.

The H_2R and β_2AR, also expressed on neutrophil granulocytes, are some of the best characterized aminergic GPCRs. Classically, both, the H_2R and the β_2AR couple to G_s proteins in order to activate adenylyl cyclases (AC) resulting in increased intracellular cAMP turnover (Fig. 3.1). Nevertheless, the β_2AR can also "signal" *via* G_i proteins, G_q proteins and β-arrestin, triggering responses distinct from those activated through G_s proteins (Wenzel-Seifert and Seifert, 2000; Seifert and Dove, 2009; Evans *et al.*, 2010). Signaling of the H_2R can also be G_q-mediated (Kühn *et al.*, 1996; Wang *et al.*, 2000). Therefore, because of the above-mentioned promiscuous coupling to G proteins, a multiplicity of signaling pathways can be activated *via* H_2Rs and β_2ARs. To make the situation even more complicated, this promiscuous signaling varies with different cell types, and various ligands are able to selectively direct the signaling in the same test system (Evans *et al.*, 2010).

As H_2R and β_2AR agonists inhibit fMLP-stimulated $O_2^{\bullet-}$ production on neutrophil granulocytes, they harbor a potential as anti-inflammatory agents (Burde *et al.*, 1990; Mirza *et al.*, 2002). The aim of the present study was the characterization of the H_2R and the β_2AR on human neutrophil granulocytes with a series of standard H_2R and β_2AR ligands, respectively (Fig. 3.2). Neutrophil granulocytes can be considered as a cell test system of high physiological relevance. cAMP accumulation assays and $O_2^{\bullet-}$ production assays were

chosen as read-outs. Measurement of the cAMP content in neutrophils is an event rather proximal to ligand-receptor interaction, whereas monitoring of H_2R- and β_2AR-mediated effects on fMLP-stimulated $O_2^{\bullet-}$ production ($O_2^{\bullet-}$ assay) is a rather indirect way of characterizing the above-mentioned receptors (Fig. 3.1). Hence, the $O_2^{\bullet-}$ assay is more susceptible to potential modulation by intracellular signaling events. Nevertheless, such downstream modulations of ligand-induced *in vivo* effects are in general of high clinical relevance, e.g. responsible for side effects of drugs, and should always be taken into consideration, when new ligands are developed. Therefore, the $O_2^{\bullet-}$ assay, in combination with the cAMP assay, enabled deeper insight into the behavior of selected receptors on neutrophil granulocytes and rendered the direct comparison of the H_2R and the β_2AR possible. In this work, we report on functional selectivities of examined H_2R and β_2AR agonists. Moreover, as repeatedly reported in the literature for other test systems (Seifert *et al.*, 1998; Wenzel-Seifert *et al.*, 2001; Baker *et al.*, 2003; Baker, 2008), on neutrophil granulocytes hH_2Rs and $h\beta_2ARs$ have distinct pharmacological profiles. And last but not least, possible reasons for the unexpected divergence in determined pK_B values for H_2R antagonists are discussed.

3.2 Materials and Methods

3.2.1 Materials

Histamine (HA), amthamine (AMT), 5-methylhistamine (5-MHA), tiotidine (TIO) and thioperamide (THIO) were obtained from Tocris Bioscience (Avonmouth, Bristol, UK). Mepyramine (MEP), dimaprit (DIM), famotidine (FAM), zolantidine (ZOL), (-)-isoproterenol (ISO), (-)-adrenaline (ADR), (±) salbutamol (SAL) and (±) dobutamine (DOB) were from Sigma-Aldrich Chemie (Steinheim, Germany). (-)-ephedrine (EPH) was from Mallinckrodt (St. Louis, MO, USA) and (±) dichlorisoproterenol (DCI) from Aldrich (Milwaukee, WI, USA). Impromidine (IMP) (Durant *et al.*, 1978) was synthesized in our laboratory. Chemical structures of ligands are depicted in Fig. 3.2. Stock solutions of HA, DIM, AMT, 5-MHA, IMP, MEP, THIO, ZOL (10 mM each) and TIO (5 mM) were prepared in Millipore water. Stock solution of FAM (2 mM) was prepared in 2 mM HCl. Stock solutions of ISO, ADR, SAL, DOB, EPH and DCI (10 mM each) were prepared in 1 mM HCL. Dilution series of all ligands were prepared in Millipore water.

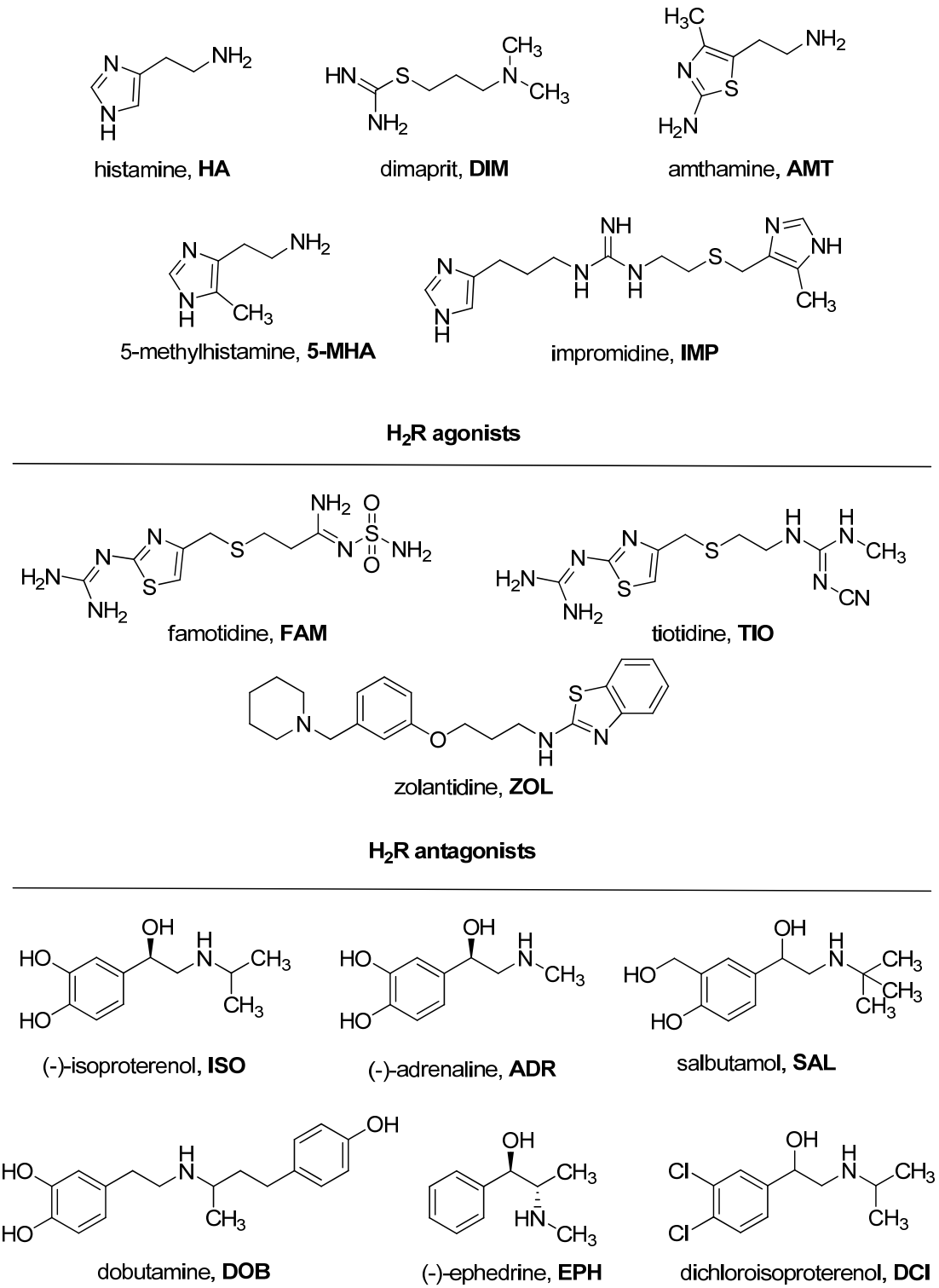

Fig. 3.2 Structures of the examined H_2R and β_2AR ligands.

10 x Dulbecco's PBS (DPBS) without Ca^{2+} and Mg^{2+} (pH 6.5 – 7.0) was purchased from PAN Biotech (Aidenbach, Germany) and Biocoll separating solution from Biochrom (Berlin, Germany). Trypan blue solution, ferricytochrome c, cytochalasin B, fMLP and IBMX were from Sigma-Aldrich Chemie (Steinheim, Germany). Solvents for extraction and HPLC analysis were purchased as follows: HPLC-gradient grade water and methanol from J. T. Baker (Deventer, The Netherlands), ammonium acetate from Sigma-Aldrich Chemie (Steinheim, Germany) and acetic acid from Riedel-de Haen (Hannover-Seelze, Germany). Tenofovir was obtained from the National Institutes of Health (Bethesda, MD, USA) and cAMP (> 99 %) from Biolog Life Science Institute (Bremen, Germany).

All reagents, used for the reverse transcription of RNA, were obtained from Fermentas (St. Leon-Rot, Germany). For PCR the DNA primers were purchased from MWG Biotech (Ebersberg, Germany). Phusion High-Fidelity DNA Polymerase and Phusion HF buffer were obtained from Finnzymes (Espoo, Finland). Sequencing was performed by GATC Biotech (Konstanz, Germany).

3.2.2 Isolation of human neutrophils

Human neutrophils were isolated from venous blood of healthy volunteers of either sex (1.6 mg EDTA/ml blood as anticoagulant) or from buffy coat preparations, obtained from the Institute for Transfusion Medicine (Medical School of Hannover, Germany). All isolation steps were carried out at room temperature. Firstly, 7 ml of venous blood or 5 ml of buffy coat was diluted to 35 ml with 1 x DPBS and carefully layered onto 15 ml of Biocoll separating solution (density 1.077 g/ml) in a 50 ml-Falcon tube. Following centrifugation (30 min, 400 x g), the upper three layers were removed. The residual pellet (~2 ml), which contained erythrocytes and granulocytes, was re-suspended in 18 ml of Millipore water and incubated for 1 min under gentle agitation in order to achieve selective lysis of the erythrocytes. Afterwards, isotonicity was restored by adding 2.2 ml of 10 x DPBS and centrifugation at 300 x g for 5 min followed. The lysis step was repeated once to remove residual erythrocytes. The cell pellet was re-suspended in 5 ml of 1 x DPBS and sedimented by centrifugation at 300 x g for 5 min. Normally, the resulting cell preparation consisted of viable neutrophils (> 98 %), as assessed by the trypan blue exclusion test. Finally, neutrophils were suspended in 1 x PBS (1 x 10^6 cells/ml for the superoxide anion assay or 1 x 10^7 cells/ml for the determination of cAMP) and stored on ice until use. Experiments were performed within 4 h after completion of isolation.

3.2.3 Superoxide anion generation ($O_2^{\bullet-}$ assay)

Reactions were carried out in 96-well plates in triplicate. Standard reaction mixtures (total volume 200 µl) contained 1 mM $CaCl_2$, 100 µM ferricytochrome c, 0.3 µg/ml cytochalasin B, examined ligand at different concentrations and 1 x 10^5 neutrophils in 1 x DPBS. After pre-incubation of the reaction mixtures for 3 min at 37 °C, reactions were initiated by addition of stimulatory fMLP (1 µM). Reference samples contained all components listed above except for fMLP. $O_2^{\bullet-}$ formation was continuously measured by monitoring the reduction of ferricytochrome c at 550 nm for 30 min at 37 °C, using a Synergy 4 microplate reader (BioTek Instruments, Winooski, VT, USA). The difference in absorbance at 550 nm between 0 min (addition of fMLP) and 30 min was used for subsequent data analysis, in order to assess agonistic activity of examined ligands. With the exception of DOB, all examined ligands did neither reduce ferricytochrome c nor stimulate $O_2^{\bullet-}$ production *per se* (data not shown). As at DOB concentrations higher than 500 nM, ferricytochrome c reduction took place, the maximum concentration of DOB used in the $O_2^{\bullet-}$ assays was 500 nM.

3.2.4 cAMP accumulation and extraction from neutrophils (cAMP assay)

Reactions were conducted in triplicate in 1.5 ml Eppendorf reaction vessels in a total volume of 100 µl. First of all, 50 µl of the reaction mixture containing $CaCl_2$ (1 mM final concentration after addition of neutrophils), IBMX (non-selective phosphodiesterase inhibitor; 100 µM) and the respective ligand at different concentrations in 1 x DPBS were pre-incubated for 5 min at 37 °C. Isolated neutrophils suspended in 1 x DPBS were pre-incubated separately for 10 min at 37 °C. Following the addition of 50 µl of neutrophils (5 x 10^5 cells/reaction vessel) to reaction mixture, samples were incubated for 10 min at 37 °C. Afterwards, samples were incubated for 10 min at 95 °C in order to stop the enzymatic reaction and then cooled to 4 °C. One hundred µl of ice-cold internal standard (tenofovir; 100 ng/ml) in eluent A (3/97 MeOH/H_2O, 50 mM NH_4OAc, 0.1 % HOAc) were added. The suspension was centrifuged at 20.800 x g at 4 °C for 5 min in order to remove denatured proteins. The cAMP concentration of the supernatant was determined by reversed phase-coupled HPLC-MS/MS.

3.2.5 Quantitation of cAMP by HPLC-MS/MS

The chromatographic separation was performed on an Agilent 1100 Series HPLC System (Agilent Technologies) equipped with a binary pump system and with a 100 µl sample loop. A combination of Supelco Column Saver (2.0 µm filter, Supelco Analytical, Bellafonte, CA, USA), Security Guard Cartridge (C18, 4 x 2 mm) in an Analytical Guard

Holder KJO-4282 (Phenomenex, Aschaffenburg, Germany) and an analytical Zorbax Eclipse XDB-C16 column (50 x 4.6 mm, 1.8 µm particle size, Agilent Technologies, Santa Clara, CA, USA), temperature controlled by a HPLC column oven at 25 °C, were used. The binary pump system supplied eluent A (50 mM ammonium acetate and 0.1 % (v/v) acetic acid in a methanol/water mixture (3/97 (v/v)) and eluent B (50 mM ammonium acetate and 0.1 % (v/v) acetic acid in a methanol/water mixture (97/3 (v/v)). The injection volume was 50 µl and the flow rate of 0.4 ml/min remained constant throughout the chromatographic run. From 0 to 5 min, the gradient of eluent B was linearly increased from 0 to 50 % of eluent B, and re-equilibrium of the column to 100 % of eluent A was achieved from 5 to 8 min. Retention times of the analyte cAMP and the internal standard tenofovir were 6.2 and 5.4 min, respectively (Fig. 3.3). The internal standard was used to mathematically correct the loss of cAMP during preparation as well as possible variabilities in HPLC-MS/MS measurement.

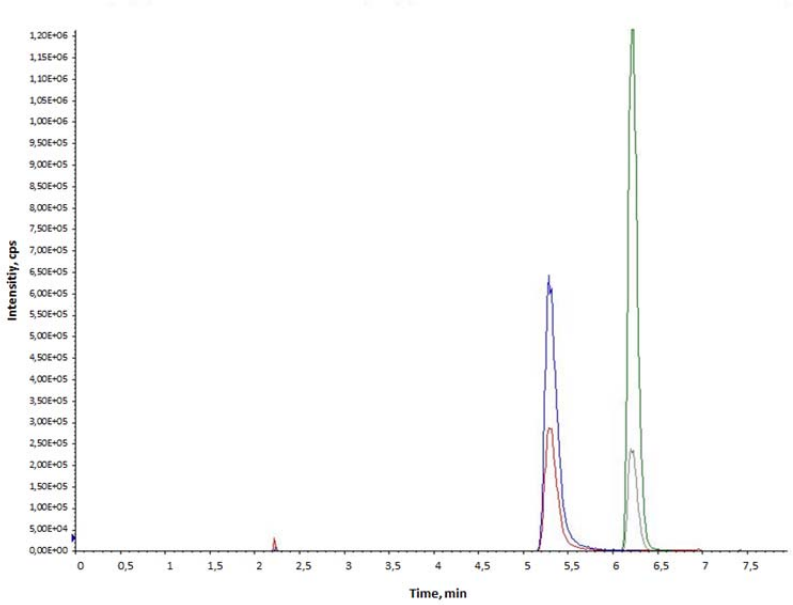

Fig. 3.3 Representative chromatogram of tenofovir (340 pmol/ml) and cAMP (320 pmol/ml) after HPLC-MS/MS detection. Blue peak, tenofovir transition +288/176 (quantifier); red peak, tenofovir transition +288/159 (qualifier); green peak, cAMP transition +330/136 (quantifier); grey peak, cAMP transition +330/312 (qualifier).

Analyte detection was conducted on an AB Sciex QTRAP 5500 triple quadropole mass spectrometer (AB Sciex, Foster City, CA, USA) using selected reaction monitoring (SRM) analysis in positive ionization mode. For this purpose nitrogen was used as collision gas. Using a 50 ms dwell time, SRM transitions were monitored as follows: cAMP +330/136 and +330/312, tenofovir +288/176 and +288/159 (Fig. 3.3). The transition +330/136 was the most intense transition of cAMP and therefore used for quantification. Additionally the +330/312 transition of cAMP was used as qualifier. The transition +288/176 of tenofovir was

used as quantifier and the transition +288/159 as qualifier. The mass spectrometer parameters were as follows: ion source voltage: 4500 V, ion source temperature: 600 °C, curtain gas: 30 psi and collision gas: 9 psi.

cAMP in samples was quantified by applying the standard curve, obtained by analysis of known amounts of pure cAMP at: 0.0262, 0.066, 0.164, 0.41, 1.024, 2.56, 6.4, 16, 40, 100, 250 pmol/tube.

3.2.6 mRNA isolation from neutrophil granulocytes, reverse transcription PCR, cDNA amplification and sequencing

Neutrophil granulocytes were isolated as described in section 3.2.2, though under sterile conditions. Total RNA was isolated from neutrophil granulocytes (7.8 x 10^8 cells) using NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The RNA content was determined with a Nanodrop 1000 Spectrometer (Thermo Scientific, Wilmington, DE, USA). Purified RNA was then used immediately for the synthesis of single-stranded cDNA (cDNA) or stored at -80 °C until use. For the synthesis of cDNA, each reaction mixture (final volume 20 µl) contained 3 µg of RNA, 0.5 µg of Oligo $(dT)_{18}$, 2 mM of dNTP Mix, 20 units of RevertAid M-MULV Reverse Transkriptase and 1 x supplied PCR buffer. RNA and Oligo$(dT)_{18}$ were denatured at 65 °C for 5 min prior to transcription (60 min, 37 °C). Finally, to inactivate enzymes, the reaction mixtures were incubated at 95 °C for 2 min.

For the amplification of cDNA by PCR two pairs of primers were used in order to discriminate between isoform 1 and isoform 2 of the hH_2R. mRNA sequences of the isoform 1 (mRNA accession number NM_001131055.1, http://www.ncbi.nlm.nih.gov/nuccore/NM_001131055.1) and the isoform 2 (mRNA accession number NM_022304.2, http://www.ncbi.nlm.nih.gov/nuccore/NM_022304.2) from NCBI database were used for the design of the primers. The forward primer hH_2R_F (5'-GATGGCACCCAATGGCACA-3') was devised to align non-selectively in the region of the START codon of both H_2R isoforms (Fig. 3.4). The reverse primer hH_2R_R_Iso1 (5'-CATCATAATTCCTGGCATGTGGTGG-3') was constructed to selectively anneal to the isoform 1 sequence in the area of STOP codon (Fig. 3.4). In the same manner, the reverse primer hH_2R_R_Iso2 (5'-CTATTACCTGTCTGTGG CTCCCTG-3') was designed for selective annealing to the isoform 2 in the area of STOP codon (Fig. 3.4). Reaction mixtures (total volume 50 µl) consisted of 5 µl of reverse transcripted cDNA, 200 µM of dNTP Mix, 200 nM of selected primers, 1 unit of Phusion High-Fidelity DNA polymerase and 1 x Phusion HF buffer. In order to exclude contamination by external DNA, a control PCR reaction mixture, containing all components mentioned above except cDNA, was prepared in parallel. PCR reactions were conducted under the following

conditions: 1 min at 98 °C, 30 cycles of [10 s at 98 °C, 20 s at 70 °C, 35 s at 72 °C], at 72 °C for 8 min.

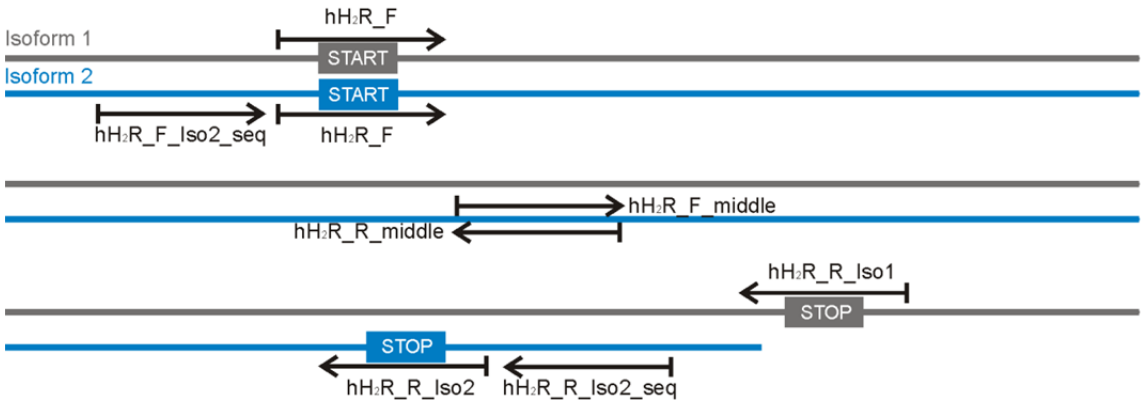

Fig. 3.4 Schematic depiction of annealing areas of selected primers on hH_2R isoform 1 and isoform 2 cDNA sequences. Expected lengths of PCR products using different primer pairs were as follows: 1197 bp with primer pair hH_2R_F/hH_2R_R_Iso1, 1085 bp with primer pair hH_2R_F/ hH_2R_R_Iso2 and 1249 bp with primer pair hH_2R_F_Iso2_seq/hH_2R_R_Iso2_seq.

In order to enable sequencing of the complete hH_2R isoform 2 between the START and the STOP codon, new primers for the isoform 2 were designed. The forward primer hH_2R_F_Iso2_seq (5'-GGCATAGTTGTCACATTGGGAGC-3') annealed to 23 base pairs (bp) upstream of the START codon of the isoform 2 and the reverse primer hH_2R_R_Iso2_seq (5'- CCAGCACCACCTAAACAGCAG-3') annealed to 21 bp of the isoform 2 downstream of the STOP codon (Fig. 3.4). Reaction mixtures (total volume 50 µl) consisted of 5 µl reverse transcripted cDNA, 200 µM of dNTP Mix, 200 nM of primers hH_2R_F_Iso2_seq and hH_2R_R_Iso2_seq each, 1 unit of Phusion High-Fidelity DNA polymerase and 1 x Phusion HF buffer. The control PCR reaction mixture contained all aforementioned components except cDNA. PCR was performed under the following conditions: 1 min at 98 °C, 38 cycles of [10 s at 98 °C, 20 s at 69 °C, 40 s at 72 °C], 8 min at 72 °C. The complete nucleotide sequence of the isoform 2 was determined by sequencing using primers hH_2R_F_Iso2_seq, hH_2R_R_Iso2_seq, hH_2R_F_middle (5'-GAAGTGTACGGG CTGGTGGATG-3') and hH_2R_R_middle (5'-CATCCACCAGCCCGTACACTTC-3') (Fig. 3.4).

3.2.7 Miscellaneous

Chromatograms, obtained by the HPLC-MS/MS analysis, were analyzed with the Analyst Software 1.5.1 (AB Sciex, Foster City, CA, USA). Data from the $O_2^{\bullet-}$ assay and the cAMP assay were analyzed with the Prism 5.01 software (GraphPad, San Diego, CA, USA). The means ± S.E.M. were always determined by the analysis of at least three independent

experiments, performed in triplicates, if not indicated otherwise. Statistical significance was always defined as $p < 0.05$ (95 % confidence interval).

3.3 Results

3.3.1 Characterisation of the H_2R on human neutrophil granulocytes with standard H_2R agonists and antagonists

In order to extensively characterize the H_2R on human neutrophil granulocytes the $O_2^{\bullet-}$ assay and the cAMP assay were used as read-outs. As already explained in the introduction, the effect of H_2R agonists can be measured as an inhibition of fMLP-stimulated $O_2^{\bullet-}$ production in the $O_2^{\bullet-}$ assay (Fig. 3.5), whereas in the cAMP assay the H_2R agonistic activity is directly proportional to an increase in cAMP accumulation (Fig. 3.6). In both cases, the inter-experimental variability was very high (Fig. 3.5C and Fig. 3.6B). Inter-individual variability of human neutrophil function had been observed previously (Seifert *et al.*, 1991).

In the $O_2^{\bullet-}$ assay, an IC_{50} value of 22.5 µM (pIC_{50} 4.65) was determined for the endogenous and standard H_2R agonist HA (Table 3.1). This value is ~2.8-fold higher than IC_{50} value (8 µM) in a similar assay format reported more than two decades ago (Burde *et al.*, 1989). We can exclude the possibility that different concentrations of cytochalasin B are responsible for this divergence (0.3 µg/ml in our $O_2^{\bullet-}$ assay and 1 µg/ml in assay from Burde *et al.* (1989)). The same group characterised HA in neutrophil granulocytes also in the absence of the cytochalasin B, and the resulting IC_{50} value was 6.7 µM (Burde *et al.*, 1990). Cytochalasin B in the $O_2^{\bullet-}$ assay has a primig role by enhancing $O_2^{\bullet-}$ formation upon exposure to fMLP (Jesaitis *et al.*, 1986; Seifert and Schultz, 1991). Nevertheless, the concentration of Ca^{2+} and Mg^{2+} in the test tubes and also handling of neutrophils during isolation and $O_2^{\bullet-}$ assay (shear forces) can influence the activation of neutrophils (Seifert and Schultz, 1991; Oh *et al.*, 2008). In our $O_2^{\bullet-}$ assay the concentration of $CaCl_2$ was identical with the $CaCl_2$ concentration in the $O_2^{\bullet-}$ assay from Burde *et al.* (1989), whereas $MgCl_2$ concentration differed between assays (0 mM and 1 mM, respectively). Additionally, instead of Biocoll separating solution, Dextran T 500 was used previously (Burde *et al.*, 1989) as a polymer for isolation of neutrophils. Therefore, besides unequal handling of neutrophils also the presence/absence of $MgCl_2$ and usage of different separation polymer could influence the extent of activation of neutrophil granulocytes and could be the reason for different potencies of HA in different test systems. Nevertheless, with respect to the potency of HA our data are comparable with the data of Ozaki and co-workers, who reported an IC_{50} value of about 30 µM for the HA-induced inhibition of the luminol-dependent chemiluminescence of granulocytes (Ozaki *et al.*, 1984).

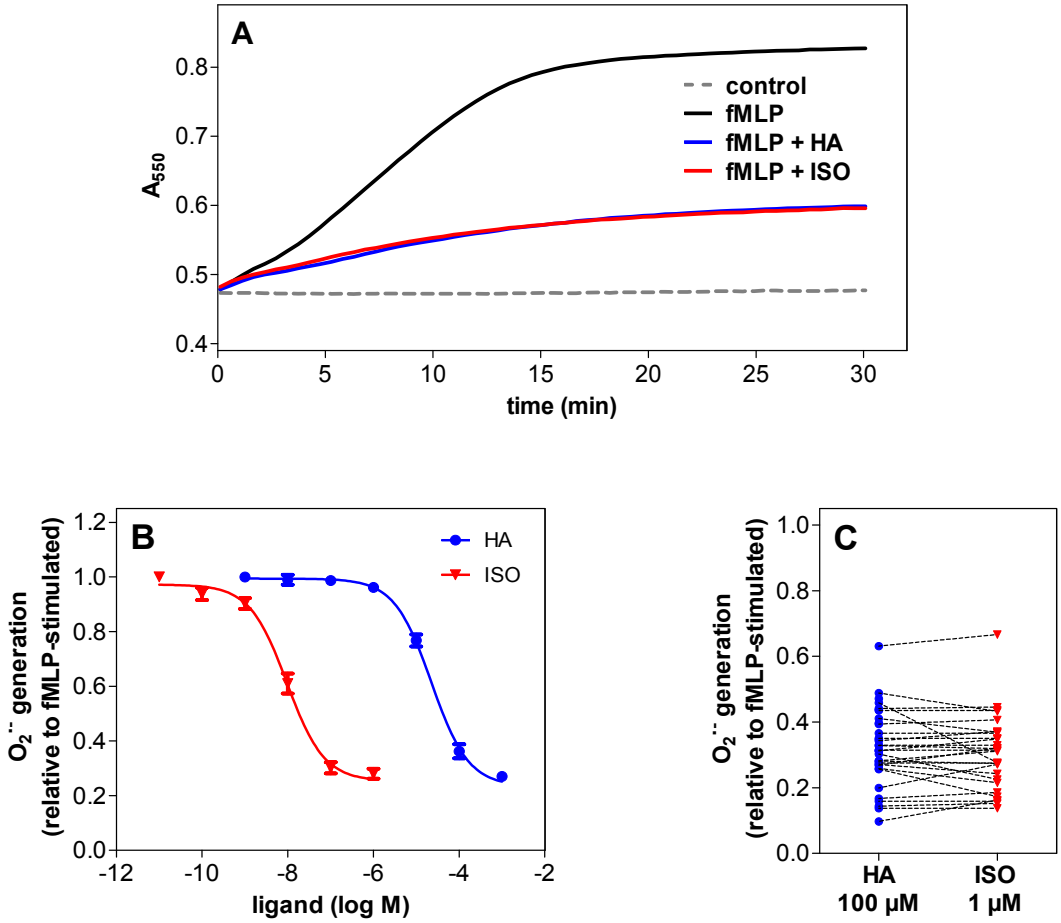

Fig. 3.5 Superoxide anion generation assay ($O_2^{\bullet-}$ assay). The $O_2^{\bullet-}$ production in human neutrophil granulocytes (1 x 10^5 cells per well) was monitored by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome c at 550 nm as described in section 3.2.3. (**A**) Continuous measurement of $O_2^{\bullet-}$ production for 30 min under control conditions (control), after stimulation with 1 µM fMLP (fMLP) and in the presence of 1 µM fMLP in combination with 100 µM HA (fMLP + HA) or 1 µM ISO (fMLP + ISO). Data shown are from one representative experiment performed in triplicate. (**B**) Concentration-response curves for HA and ISO in the $O_2^{\bullet-}$ assay. Data shown are from eight (HA) or nine (ISO) independent experiments, performed in triplicate (data points are means ± S.E.M.). Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curve. (**C**) Inter-experimental variability of inhibitory effect of 100 µM HA and 1 µM ISO on fMLP-stimulated $O_2^{\bullet-}$ production. Each data point represents one independent experiment. Dashed lines connect data points obtained in one experiment. Increase in absorbance at 550 nm during 30 min after addition of fMLP was set to 1.00 and increase in absorbance in the presence of 100 µM HA (+ fMLP) or 1 µM ISO (+ fMLP) in each assay was compared to this value.

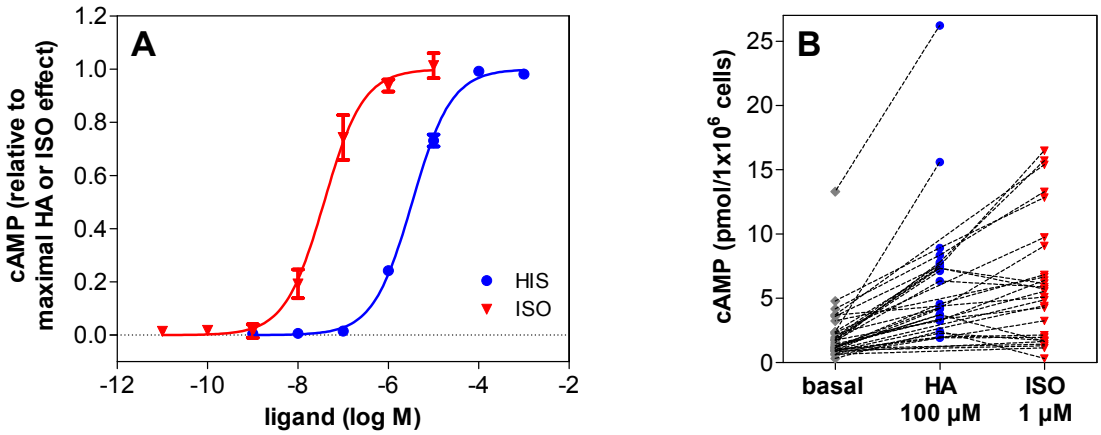

Fig. 3.6 Measurement of cAMP content in neutrophil granulocytes (cAMP assay). The cAMP level in human neutrophil granulocytes (5 x 10^5 cells per sample) was monitored by HPLC-MS/MS system as described in sections 3.2.4 and 3.2.5. (**A**) Concentration-response curves for HA and ISO in the cAMP assay. Data shown are from four (ISO) to five (HA) independent experiments, performed in duplicate or triplicate (data points are mean ± S.E.M.). The maximal HA- and ISO-induced cAMP production was set to 1.00. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curve. **(B)** Inter-experimental variability of basal cAMP concentration in neutrophil granulocytes (basal) and cAMP level after stimulation with 100 µM HA (HA 100 µM) and 1 µM ISO (ISO 1 µM). Each data point represents one independent experiment. Dashed lines connect data points, obtained in one experiment.

Burde and coworkers reported IMP being a more potent H_2R agonist than HA in the $O_2^{\bullet-}$ assay with an IC_{50} value of 2 µM and with efficacy comparable to HA (Burde *et al.*, 1989). Surprisingly, in our $O_2^{\bullet-}$ test system IMP shows very weak partial agonistic activity with an IC_{50} value above 100 µM (Table 3.1). We do not have a plausible explanation for this difference. However, IMP was more potent than HA in the cAMP assay on neutrophil granulocytes, but still possessing only weak partial agonistic activity.

The potencies and efficacies of the H_2R agonists HA, DIM, AMT, 5-MHA and IMP in the $O_2^{\bullet-}$ and the cAMP assay are given in Table 3.1. Additionally, the EC_{50} and E_{max} values of ligands determined in steady-state GTPase activity assays using membrane preparations of Sf9 insect cells expressing the hH_2R-$G_{s\alpha S}$ fusion protein (Preuss *et al.*, 2007; Igel, 2009; Kraus *et al.*, 2009) are listed in Table 3.1. Comparing these data, an increase in potency is observed in the order $O_2^{\bullet-}$ assay < cAMP assay < GTPase assay for all ligands without exception. Unfortunately, the efficacy of H_2R agonists in all three test systems cannot be reliably compared because of the weak effect of DIM, 5-MHA and IMP in the $O_2^{\bullet-}$ assay. Slightly superior efficacies of DIM, AMT and 5-MHA were determined in the cAMP assay in comparison to the GTPase assay whereas the efficacy of IMP was higher in the GTPase assay.

Table 3.1 Comparison of potencies and efficacies of standard H_2R agonists, determined in three different test systems.

Cpd.	$O_2^{\bullet-}$ assay (hH_2R on neutrophil granulocytes)		cAMP assay (hH_2R on neutrophil granulocytes)		GTPase assay (recombinant protein hH_2R-$G_{s\alpha S}$)	
	pIC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}
HA	4.65 ± 0.06	1.00	5.47 ± 0.03	1.00	6.00[b]	1.00
DIM	< 4	0.60 ± 0.03[a]	4.79 ± 0.07	1.04 ± 0.04	6.04[b]	0.85 ± 0.02[b]
AMT	5.52 ± 0.07	1.06 ± 0.04	6.05 ± 0.03	0.94 ± 0.01	6.72[b]	0.91 ± 0.02[b]
5-MHA	4.21 ± 0.15	0.62 ± 0.03[a]	4.92 ± 0.07	1.09 ± 0.04	5.54[c]	1.01 ± 0.03[c]
IMP	< 4	0.10 ± 0.03[a]	6.37 ± 0.19	0.38 ± 0.04	6.80[b]	0.82 ± 0.02[b]

On human neutrophil granulocytes, the $O_2^{\bullet-}$ assay (1 x 10^5 cells per well) was performed as described in section 3.2.3 and the cAMP assay (5 x 10^5 cells per cup) was performed as described in sections 3.2.4 and 3.2.5. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are the means ± S.E.M. of three to eight independent experiments performed in triplicate. The efficacy (E_{max}) of HA in each assay was set to 1.00 and the efficacies of other ligands were referred to this value.

[a] Up to 100 µM, concentration-response curve for compound did not achieve saturation, therefore, inhibition of fMLP-stimulated $O_2^{\bullet-}$ production at fixed concentration of 100 µM is given (relative to HA).

[b] Data were taken from Preuss *et al.* (2007).

[c] Data were taken from Igel (2009).

[d] Data were taken from Kraus *et al.* (2009).

The inhibition of the fMLP-stimulated $O_2^{\bullet-}$ production by HA, AMT, DIM and 5-MHA was counteracted by the selective H_2R antagonist FAM (Fig. 3.7). The effect of 100 µM AMT was only partially antagonized by 10 µM FAM. Higher concentrations of FAM were intentionally avoided because at concentrations above 30 µM FAM *per se* inhibits fMLP-stimulated $O_2^{\bullet-}$ production (data not shown) (Burde *et al.*, 1990). Incubation of neutrophil granulocytes in the presence of the selective H_1R antagonist MEP or the H_3R/H_4R antagonist THIO did not alter the HA- or AMT-induced inhibition of $O_2^{\bullet-}$ production. In addition, we could demonstrate a H_2R-mediated stimulation of cAMP accumulation by HA (Fig. 3.8).

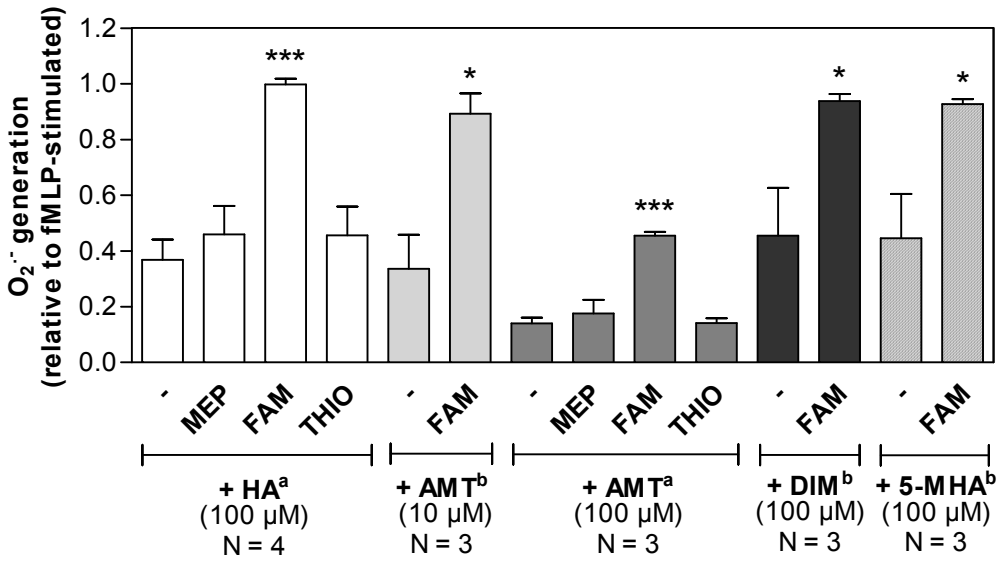

Fig. 3.7 Antagonistic effects of MEP, FAM and THIO on HA-, AMT-, DIM- and 5-MHA-induced inhibition of $O_2^{\bullet-}$ production. The $O_2^{\bullet-}$ production in human neutrophil granulocytes (1 x 10^5 cells per well) was monitored by the determination of ferricytochrome c reduction at 550 nm as described in section 3.2.3. Data shown are means ± S.E.M. of three to four independent experiments, performed in triplicate. MEP, mepyramine at a concentration of 1 µM; FAM, famotidine at a concentration of 10 µM; THIO, thioperamide at a concentration of 10 µM. [a] The inhibition of $O_2^{\bullet-}$ production by HA or AMT in the absence of antagonists (reference value) was compared with the inhibition of $O_2^{\bullet-}$ production by HA or AMT in the presence of MEP, FAM and THIO, respectively, using one-way ANOVA, followed by Dunnett's multiple comparison test (***, $p < 0.001$; 95 % confidence interval). [b] The inhibition of $O_2^{\bullet-}$ production by AMT, DIM or 5-MHA in the absence and in the presence of FAM was compared with unpaired two-tailed t-test (*, $p < 0.05$; 95 % confidence interval).

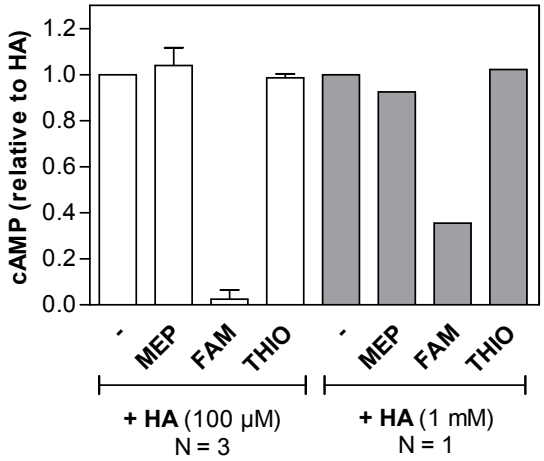

Fig. 3.8 Antagonistic effects of MEP, FAM and THIO in the cAMP assay. cAMP accumulation was monitored in neutrophil granulocytes (5 x 10^5 cells per cup) by HPLC-MS/MS system as described in sections 3.2.4 and 3.2.5. Data shown are from one or three (mean ± S.E.M.) independent experiments, performed in triplicate. MEP, mepyramine at a concentration of 1 µM; FAM, famotidine at a concentration of 10 µM; THIO, thioperamide at a concentration of 10 µM.

Because antagonistic potencies are considered constant for a specific antagonist-receptor interaction, irrespective of the test system used (Hill, 2006), we investigated the effect of H_2R antagonists on neutrophil granulocytes in more detail. First of all, the potency of FAM for H_2R in human neutrophil granulocytes was determined using Schild analysis (Schild, 1947). The parallel shift of the concentration-response curve for histamine to the right was observed by applying FAM at concentrations of 0.1, 0.3, 1 and 3 µM in the cAMP assay (Fig.

3.9A). The maximal agonist response was depressed at very high concentrations of HA (3 and 5 µM) which is due to an unspecific effect of HA at these concentrations on cAMP production, and not due to an insurmountable antagonistic activity of FAM (data not shown). A Schild plot of the data from Fig. 3.9A is shown in Fig. 3.9B. The slope of the regression line is 1.14 (95 % confidence interval from 0.83 to 1.44) and the intercept with the abscissa is -7.42, resulting in a pA_2 value of 7.42 (95 % confidence interval from 7.14 to 7.88). This pA_2 value correlates very well with the pA_2 value (7.5) for FAM determined at human neutrophil granulocytes more than two decades ago by Burde and coworkers (Burde *et al.*, 1989). Here, the HA was used as H_2R agonists as well but the $O_2^{\bullet-}$ assay was chosen as read out.

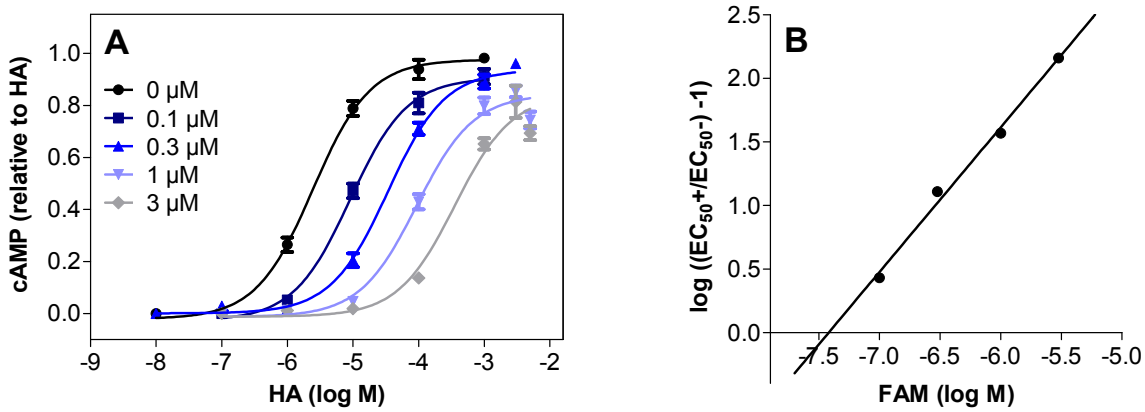

Fig. 3.9 Effect of the H_2R antagonist FAM on HA-induced cAMP production in human neutrophil granulocytes. The cAMP level in human neutrophil granulocytes (5 x 10^5 cells per sample) was monitored by HPLC-MS/MS system as described in sections 3.2.4 and 3.2.5. (**A**) The effect of FAM at fixed concentrations (0 µM, 0.1 µM, 0.3 µM, 1 µM and 3 µM) on the HA-induced cAMP production. Data shown are from two independent experiments, performed in duplicate (data points are mean ± S.E.M.). The maximal HA-induced cAMP production in the absence of FAM was set to 1.00 and all other data points were referred to this value. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curve. (**B**) Schild plot of the data shown in panel A. EC_{50}+, EC_{50} value for the HA-induced cAMP production in the presence of FAM; EC_{50}-, EC_{50} value for the HA-induced cAMP production in the absence of FAM.

Additionally, the pK_B values for FAM, TIO and ZOL were determined in the $O_2^{\bullet-}$ assay as well as in the cAMP assay by applying a submaximally effective concentration of HA in each assay and increasing concentrations of H_2R antagonists. In Table 3.2, the results are summarized and compared with literature data (GTPase assay using membranes of Sf9 insect cells expressing hH_2R-$G_{s\alpha S}$) (Kelley *et al.*, 2001). In all test systems, the pK_B values increased in the order ZOL < TIO < FAM. Nevertheless, when pK_B values of the individual H_2R antagonists in different test systems were compared, large deviations were observed. Even for the same cells, neutrophils, the pK_B values in the cAMP assay are for up to 0.9 log units higher than those determined in the $O_2^{\bullet-}$ assay.

Table 3.2 Comparison of pK_B values of the H_2R antagonists FAM, TIO and ZOL, determined in three different test systems.

Cpd.	$O_2^{\bullet-}$ assay (hH_2R on neutrophil granulocytes)	cAMP assay (hH_2R on neutrophil granulocytes)	GTPase assay (recombinant protein hH_2R-$G_{s\alpha S}$)[a]
	pK_B *(pIC_{50} ± S.E.M.)*	pK_B *(pIC_{50} ± S.E.M.)*	pK_B
FAM	7.01 *(6.68 ± 0.07)*	7.90 *(7.62 ± 0.14)*	7.54
TIO	6.08 *(5.75 ± 0.08)*	6.95 *(6.68 ± 0.17)*	7.22
ZOL	5.86 *(5.53 ± 0.06)*	6.54 *(6.27 ± 0.18)*	6.00

On human neutrophil granulocytes, the $O_2^{\bullet-}$ assay (1 x 10^5 cells per well) was performed as described in section 3.2.3 and the cAMP assay (5 x 10^5 cells per sample) was performed as described in sections 3.2.4 and 3.2.5. $O_2^{\bullet-}$ and cAMP production were determined at submaximally effective concentration of HA (25 µM and 3 µM, respectively) in the presence of increasing concentrations of H_2R antagonists. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are from four to six independent experiments performed in triplicate. The pK_B values were calculated from the IC_{50} values according to Cheng and Prusoff (Cheng and Prusoff, 1973).

[a] Data of GTPase assays (Sf9 membranes with hH_2R-$G_{s\alpha S}$ fusion protein) were taken from Kelley *et al.* (2001). The reported non-logarithmic K_B values were converted into logarithmic pK_B values.

3.3.2 Determination of H_2R isoform expression in human neutrophil granulocytes

Two isoforms of the hH_2R are described in literature. The longer isoform 1 was identified by researchers involved in the Mammalian Gene Collection Program of the National Institutes of Health (Strausberg *et al.*, 2002). This isoform encodes 397 amino acids. The isoform 2 was successfully cloned for the first time from human gastric fundic mucosa by Gantz and coworkers (Gantz *et al.*, 1991). The second isoform, which has a truncated C-terminus compared to the isoform 1, encodes a protein comprising 359 amino acids.

It is evident from the already presented results that the hH_2R in neutrophil granulocytes behaves differently from the recombinant fusion protein hH_2R-$G_{s\alpha S}$, in which isoform 2 of the hH_2R is expressed. Therefore, the H_2R isoform in human neutrophil granulocytes was determined. After isolation of total mRNA from neutrophils and reverse transcription to cDNA, primer pairs, specific for isoform 1 and isoform 2, respectively, were used for amplification of the cDNA by PCR. No PCR product was obtained for isoform 1 (expected PCR product length 1197 bp), whereas the expected 1085 bp long PCR product could be detected with an isoform 2 specific primer pair (Fig. 3.10). Sequencing of the complete encoding region confirmed the presence of isoform 2 in neutrophil granulocytes (see supplemental data in chapter 7), and this isoform has 100 % amino acid sequence identity with the isoform present in the fusion protein hH_2R-$G_{s\alpha S}$.

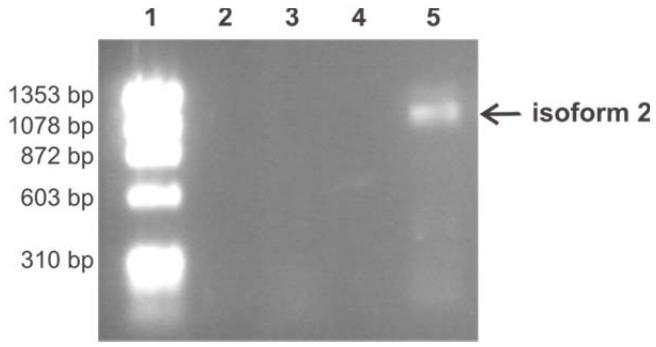

Fig. 3.10 Amplification of cDNA, reverse-transcribed from total RNA of human neutrophil granulocytes, by PCR using H_2R isoform 1- and isoform 2-specific primer pairs. Isolation of total RNA from human neutrophil granulocytes, reverse transcription PCR and amplification of cDNA were performed as described in section 3.2.6. **1**, DNA ladder with five fragments of differential length, the length of each fragment is given as number of base pairs (bp) on the left side of the figure; **2**, control PCR without cDNA as template, using the isoform 1-specific primer pair hH_2R_F and $hH_2R\_R\_Iso1$; **3**, PCR with cDNA as template, using the isoform 1-specific primer pair hH_2R_F and $hH_2R\_R\_Iso1$; **4**, control PCR without cDNA as template, using the isoform 2-specific primer pair hH_2R_F and $hH_2R\_R\_Iso2$; **5**, PCR with cDNA as template, using the isoform 2-specific primer pair hH_2R_F and $hH_2R\_R\_Iso2$.

3.3.3 Characterisation of the β_2AR on human neutrophil granulocytes with standard β_2AR agonists

Profound differences were found between the pharmacological profile of the hH_2R in recombinant test system and on human neutrophil granulocytes. In order to answer the question whether this observation is H_2R-specific or a common characteristic of G_s-coupled GPCRs, we additionally characterized the β_2AR on human neutrophil granulocytes. By analogy with the hH_2R, the β_2AR was characterized in the $O_2^{\bullet-}$ assay and in the cAMP assay using standard β_2AR agonists with efficacies varying from very weak partial to full agonism. Data from our study and literature data from GTPase assays using membrane preparations of Sf9 insect cells expressing $h\beta_2AR\text{-}G_{s\alpha S}$ (Seifert *et al.*, 1998; Weitl and Seifert, 2008) are summarized in Table 3.3.

Potencies of ISO, ADR, SAL and DOB were higher in the $O_2^{\bullet-}$ assay than in the cAMP assay. EPH and DCI were lacking any agonistic activity in the cAMP assay at concentrations up to 100 µM. The efficacies of ADR and DOB were comparable in both test systems, but the efficacy of SAL was superior in the $O_2^{\bullet-}$ assay. Detailed analysis of data from $O_2^{\bullet-}$ assay, cAMP assay and GTPase assay can be summarized as follows. In general, the rank order of potency was cAMP assay < GTPase assay < $O_2^{\bullet-}$ assay whereas the rank order of efficacy was cAMP assay < $O_2^{\bullet-}$ assay ≈ GTPase assay.

Table 3.3 Comparison of potencies and efficacies of standard β_2AR agonists, determined in three different test systems.

Cpd.	$O_2^{\bullet-}$ assay (β_2AR on neutrophil granulocytes)		cAMP assay (β_2AR on neutrophil granulocytes)		GTPase assay (recombinant protein hβ_2AR-$G_{s\alpha S}$)	
	pIC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}
ISO	8.02 ± 0.07	1.00	7.42 ± 0.10	1.00	7.50[b]	1.00[b,c]
ADR	7.82 ± 0.09	1.01 ± 0.05	6.81 ± 0.09	1.06 ± 0.04	7.37[c]	1.00[c]
SAL	7.16 ± 0.12	0.77 ± 0.04	6.74 ± 0.15	0.35 ± 0.03	6.70[b]	0.74 ± 0.04[b]
DOB	7.90 ± 0.41[a]	0.15 ± 0.03[a]	4.86 ± 0.36	0.21 ± 0.05	6.70[b]	0.45 ± 0.07[b]
EPH	5.95 ± 0.23	0.34 ± 0.04	< 4	0.01 ± 0.01	4.69[b]	0.31 ± 0.02[b]
DCI	4.40 ± 0.26	0.32 ± 0.06	< 4	0.00 ± 0.01	7.09[b]	0.17 ± 0.06[b]

On human neutrophil granulocytes, the $O_2^{\bullet-}$ assay (1 x 10^5 cells per well) was performed as described in section 3.2.3 and the cAMP assay (5 x 10^5 cells per cup) was performed as described in sections 3.2.4 and 3.2.5. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are the means ± S.E.M. of four to nine independent experiments performed in triplicate. The efficacy (E_{max}) of ISO in each assay was set to 1.00 and the efficacies of other ligands were referred to this value.

[a] The highest concentration used was 500 nM. DOB at concentrations higher than 500 nM causes reduction of ferricytochrome c *per se*.

[a] Data were taken from Seifert *et al.* (1998). The reported non-logarithmic EC_{50} values were converted into logarithmic pEC_{50} values.

[c] Data were taken from Weitl and Seifert (2008). The non-logarithmic EC_{50} value was converted into logarithmic pEC_{50} value.

3.4 Discussion

Drugs targeting hH_2Rs and β_2ARs have been used for decades in the therapy of humans. H_2R antagonists like famotidine and ranitidine have been widely used in the treatment of gastroesophaegal reflux disease and peptic ulcer, whereas H_2R agonistic activity is beneficial in the therapy of acute myeloid leukemia (van der Goot and Timmerman, 2000; Martner *et al.*, 2010). Numerous short- and long-acting β_2AR agonists are indispensable as bronchodilatators in patients with asthma or chronic obstructive pulmonary disease (Mansfield, 2008). Despite the great success of the established therapies based on H_2R- and β_2AR-mediated effects, these receptors are still in the focus of numerous projects. Among others, one reason is the wide distribution of both receptors in different tissues, rendering it possible to employ new therapies or to reduce side effects of the existing ones. For example, data from literature suggest a potential use of H_2R and β_2AR agonists as anti-inflammatory agents (Burde *et al.*, 1990; Mirza *et al.*, 2002). Therefore, we decided to perform a thorough characterization of both receptors on human neutrophil granulocytes using two distinct read-outs. Moreover, the parallel characterization of both receptors on neutrophil granulocytes allowed us their direct comparison.

3.4.1 Indications for functional selectivity of H_2R and β_2AR agonists

The selectivity of GPCRs for different signaling pathways depends on more factors. One very important aspect is a ligand-directed trafficking of receptor signaling, also termed functional selectivity, ligand bias, biased agonism or stimulus trafficking in literature (Galandrin *et al.*, 2007). All these terms stand for the ability of ligands to differentially activate distinct signaling pathways by stabilizing ligand-specific conformations of the same receptor protein. Functional selectivity has already been reported for numerous GPCRs such as dopamine D_1 and D_2 receptors, the histamine H_3 receptor, adenosine A_1 and A_3 receptors, the α_{2A}-adrenoceptor and the β_2AR (Kenakin and Miller, 2010). Biased ligands can differently activate G protein-dependent and -independent signaling such as the β-arrestin pathway (Azzi *et al.*, 2003; Rosethorne and Charlton, 2011), can discriminate between G_s, G_i, G_q and other G protein-mediated routes (Seifert and Dove, 2009) or even selectively modulate e.g. G_{i1}, G_{i2} and G_{i3} protein subtype activities (Mukhopadhyay and Howlett, 2005).

Therefore, it is not surprising that any given ligand possesses multiple potencies and efficacies depending on the down-stream pathway analyzed (Kenakin and Miller, 2010). This observation was also confirmed in our study with H_2R and β_2AR agonists using the $O_2^{\bullet-}$ assay and the cAMP assay as read-outs and by comparison of the results with literature data, obtained in GTPase assays on Sf9 membranes expressing hH_2R-$G_{s\alpha S}$ and $h\beta_2AR$-$G_{s\alpha S}$ (Fig. 3.11). We could rule out the possibility that different receptor isoforms are the reason for the divergence in potency and efficacy of H_2R agonists between hH_2R on neutrophils and hH_2R-$G_{s\alpha S}$ fusion protein, expressed in Sf9 insect cells (see section 3.3.2). The potency of all H_2R agonists clearly decreased in direction GTPase assay > cAMP assay > $O_2^{\bullet-}$ assay whereas the efficacy profile of the same ligands was not uniform. For example, the efficacy of DIM is higher in the cAMP assay than in the GTPase assay. In contrast, the efficacy of IMP is more than twofold higher in the GTPase assay. By analogy, the efficacy of ADR is comparable in all three test systems, whereas the efficacy of SAL is drastically decreased in the cAMP assay and the efficacy of DOB substantially increased in the GTPase assay, compared to the other two test systems. Moreover, DCI is less potent in the $O_2^{\bullet-}$ assay than in the GTPase assay, in contrast to other β_2AR agonist. Collectively, these data suggest that examined agonists are able to stabilize distinct receptor conformations of targeted receptors supporting the concept of functional selectivity.

It should be noted that the vast majority of reports about functional selectivity in the literature originates from recombinant test systems, where receptor-G protein coupling can be more easily controlled (Kenakin and Miller, 2010), e.g. by expression of only receptor and G-proteins of interest. On the contrary, the investigation of functional selectivity in neutrophil granulocytes is hampered by limited possibilities to block coupling to G proteins; G_i proteins can be normally switched off with pertusis toxin, whereas there is no pharmacological tool

available for the selective inhibition of G_s and G_q coupling. However, neutrophils enable - although in a limited manner - the investigation of functional selectivity under more relevant physiological conditions.

Moreover, our results demonstrate once more a need for the characterization of compounds in numerous *in vitro* and *in vivo* test systems at an early stage of development before extensive studies on patients are planned in order to avoid withdrawal of clinical studies because of unexpected drug effects. Additionally, the usage of a broad spectrum of test systems can be awarded with identification of additional beneficial effects of drugs. For example, selective activation of β_2AR-mediated G_s-coupled signaling in rat cardiomyocytes by fenoterol had beneficial effects in congestive heart failure (Woo *et al.*, 2009). Moreover, the long-acting β_2AR agonist salmeterol showed beneficial anti-inflammatory effects in the lungs of mice by inhibiting lipopolysaccharide-induced influx of neutrophil granulocytes into bronchoalveolar lavage fluid (Maris *et al.*, 2004).

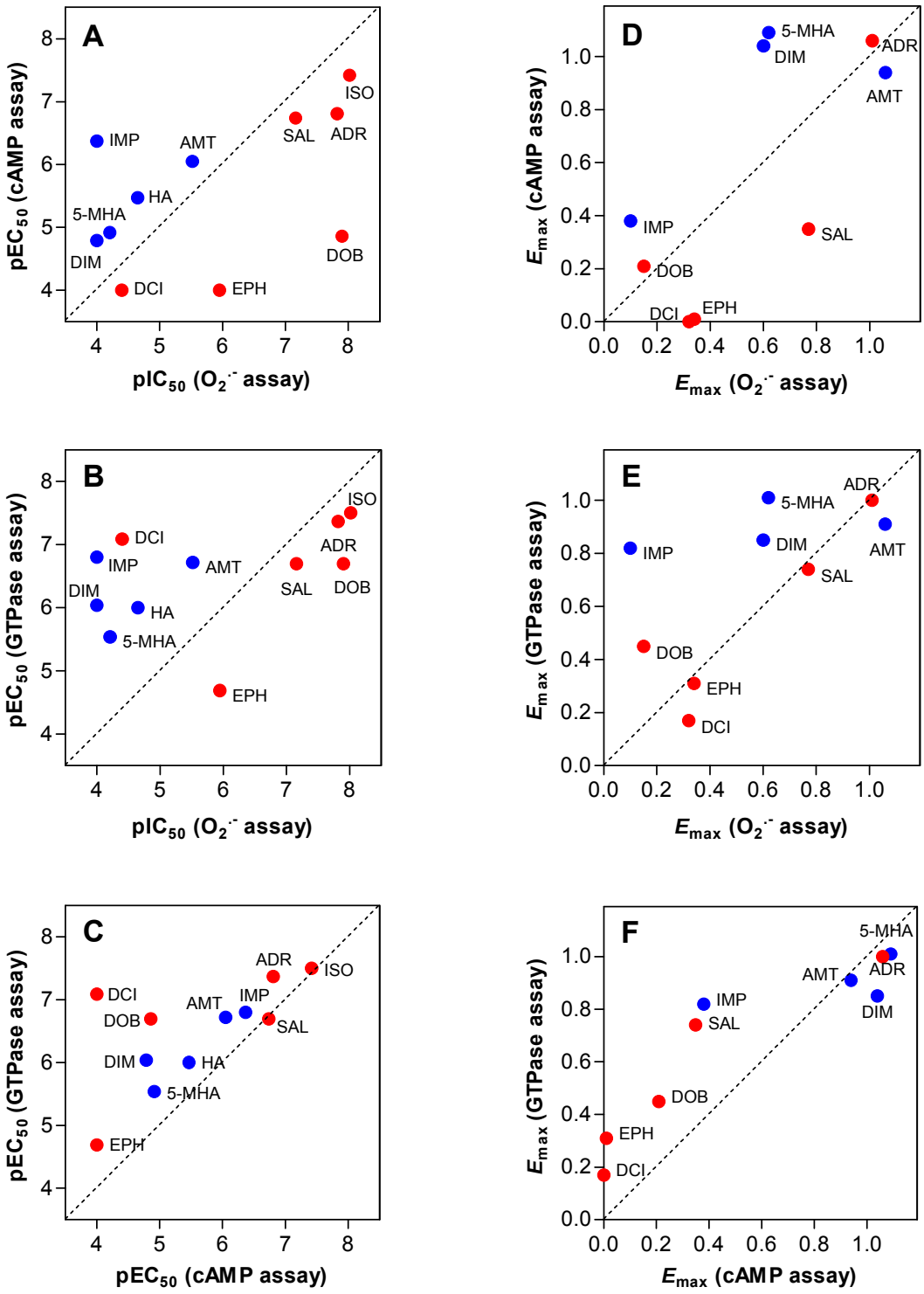

Fig. 3.11 Pair-wise direct comparison of the potencies (A-C) and efficacies (D-E) of H_2R and β_2AR agonists in the $O_2^{\bullet-}$ assay, the cAMP assay and the GTPase assay. Data for H_2R agonists were taken from Table 3.1, whereas data for β_2AR agonists are from Table 3.3. H_2R agonists are represented as blue circles and β_2AR agonists as red circles. The dotted line indicates pharmacological identity of the two parameters compared.

3.4.2 Differing properties of hH_2Rs and β_2ARs on neutrophil granulocytes

Substantial differences between the hH_2R and the $h\beta_2AR$ have been reported repeatedly in the literature although both receptors classically couple to G_s proteins. The $h\beta_2AR$ fused to the long splice variant of the $G_{s\alpha}$ protein ($G_{s\alpha L}$) possessed higher apparent constitutive activity than $h\beta_2AR$ fused to the short splice variant $G_{s\alpha S}$ (Seifert *et al.*, 1998). By contrast, the hH_2R fused to $G_{s\alpha L}$ and $G_{s\alpha S}$ exhibited similar apparent constitutive activity (Wenzel-Seifert *et al.*, 2001). Moreover, a decrease in potency for all examined H_2R agonists was observed in cAMP response element (CRE)-mediated reporter gene transcription assay in comparison with the cAMP accumulation assay on CHO cells, stably expressing hH_2R (Baker, 2008). Using similar experimental conditions for the characterization of the $h\beta_2AR$ on CHO cells by the same research group, high-efficacy agonists appeared more potent in the cAMP assay and less efficacious agonists more potent in CRE reporter gene assay (Baker *et al.*, 2003).

By analogy with aforementioned data, obtained in recombinant test systems, the H_2R and the β_2AR behaved differently also on human neutrophil granulocytes. Extremely low potency of H_2R agonists on the one hand and clearly higher potency of β_2AR agonists on the other hand was determined in the $O_2^{\bullet-}$ assay compared to the cAMP assay. Is has been already mentioned in the introduction that β_2ARs can couple to G_s, G_i and G_q proteins whereas H_2R was reported to couple to G_s and G_q proteins. Although both receptors on neutrophils effectively couple to G_s resulting in increased cAMP turnover we cannot exclude the possibility that subtle G_i- or G_q-mediated effects interfere with fMLP-stimulated $O_2^{\bullet-}$ production. The second and the third intracellular loop, essential for coupling to G proteins (Kobilka, 1992), differ between hH_2R and $h\beta_2AR$ (Wang *et al.*, 2000). In an elegant study of Wong and coworkers, the replacement of the second and the third intracellular loop of the $h\beta_2AR$ with their counterparts from the hH_2R decreased the ability of the $h\beta_2AR$ to activate AC but increased the activation of PLC/PKC signaling pathway in HEK 293 cells (Wang *et al.*, 2000). One could speculate that additional coupling of the hH_2R to G_q protein in neutrophil granulocytes leads to activation of the PLC/PKC signaling pathway resulting in increased $O_2^{\bullet-}$ production. However, an early study of Leino and coworkers negated this speculation by reporting that HA *via* H_2R actually inhibits Ca^{2+} increase induced by fMLP (Leino *et al.*, 1993).

Moreover, different compartmentation of GPCRs and their signaling partners at the cell membrane in microdomains like lipid rafts or caveolae can modulate subcellular signaling events (Ostrom and Insel, 2004; Lasley, 2011). β_2AR, G_s, G_i, G_q, some AC types and PLC isoforms were localized in lipid rafts and caveolae of cardiac myocytes or membranes (Insel *et al.*, 2005). Lipid rafts were identified also on the plasma membrane of bovine and human neutrophil granulocytes (Nebl *et al.*, 2002; Shao *et al.*, 2003). Moreover, Shao and coworkers

showed that membrane-bound components of NADPH-oxidase are localized in detergent insoluble lipid rafts. Upon neutrophil stimulation, cytosolic components of NADPH-oxidase and PKC were additionally recruited to the lipid rafts improving the coupling efficiency by juxtapose of signaling partners (Shao *et al.*, 2003). Therefore, co-localisation of β_2AR signaling partners could favor efficient cAMP accumulation and additional subsequent signaling events resulting in very effective inhibition of fMLP-stimulated $O_2^{\bullet-}$ production in neutrophils. However, in case of the hH_2R, the sufficient activation of ACs but weak signaling transmission because of the inappropriate localization of downstream signaling partners could be an explanation for very modest inhibition of fMLP-stimulated $O_2^{\bullet-}$ production by H_2R agonists.

Gong and coworkers reported that ISO activated NADPH oxidase *via* β-arrestin-1-dependent way using knockdown of β-arrestin-1 by RNA interference in HEK293 cells transfected with $h\beta_2$AR (Gong *et al.*, 2008). Activation of cAMP/PKA-independent inhibitory signaling pathways could be a plausible explanation for enhanced potencies of β_2AR agonists in the $O_2^{\bullet-}$ assay compared to the cAMP assay on human neutrophil granulocytes. Unlike in the case of β_2AR agonists, lower potencies of H_2R agonists in the $O_2^{\bullet-}$ assay than in the cAMP assay could be connected with the ability of the hH_2R to trigger solely cAMP/PKA-dependent inhibition of $O_2^{\bullet-}$ production. However, additional studies with β_2AR antagonists and PKA inhibitors are needed to investigate this possibility. Preliminary data of ongoing study indicate that β_2AR antagonists are able to antagonize $h\beta_2$AR-mediated $O_2^{\bullet-}$ production in neutrophils (data not shown).

On the first glance, the lack of agonistic activity of EPH and DCI as well as the weak partial agonistic activity of SAL in the cAMP assay are surprising. In the $O_2^{\bullet-}$ assay, the coupling efficacy of the named ligands increases by 32 - 42 % relative to ISO. One plausible explanation would be that partial agonists desensitize β_2ARs to a lower extent than highly efficacious ISO and ADR resulting in improved effectiveness of partial agonists in downstream inhibition of fMLP-stimulated $O_2^{\bullet-}$ production relative to full agonists. The correlation between the strength of β_2AR agonist coupling and agonist-induced desensitization, internalization and phosphorylation has already been reported before (January *et al.*, 1997; Clark *et al.*, 1999; Baker *et al.*, 2003). Unfortunately, direct comparison of H_2R agonist efficacies between the $O_2^{\bullet-}$ assay and the cAMP assay was unfeasible because concentration-response curves for DIM, 5-MHA and IMP up to concentrations as high as 100 µM did not achieve saturation in the $O_2^{\bullet-}$ assay.

It should be kept in mind that kinetics of the GPCR-G protein coupling and kinetics of coupling to further signaling partners (Wenzel-Seifert and Seifert, 2000), different expression levels of receptors as well as potential formation of homo- and heteromers (Hebert *et al.*, 1996; Fukushima *et al.*, 1997; Smith and Milligan, 2010) also add to the

complexity of β_2AR and H_2R behavior on neutrophil granulocytes. Evidently, characteristics of one GPCR, that classically couples to G_s, cannot be interpreted as common characteristic of all G_s coupled GPCRs, although the same test system is used for the characterization.

3.4.3 Unexpected effects of H_2R antagonists on neutrophil granulocytes

According to the established understanding of the antagonist-GPCR interaction, potency of an antagonist for a given receptor is a constant irrespective of the tissue selected as a test system, the agonist used for the stimulation of GPCR and downstream signaling event monitored. Of course a prerequisite is that no chemical modification of the antagonist or GPCR occurs (Baker *et al.*, 2003; Hill, 2006). Very interesting studies on this topic were performed by Baker and coworkers using chinese hamster ovary (CHO) cells stably expressing hβ_2AR and hH_2R, respectively (Baker *et al.*, 2003; Baker, 2008). By monitoring the cAMP accumulation (incubation time 10 min) and CRE-mediated reporter gene transcription (incubation time 5 hours) different antagonist affinities were determined for the examined β_2AR antagonists ICI 118,551 ((2*R**,3*S**)-3-(isopropylamino)-1-(7-methyl-2,3-dihydro-1*H*-inden-4-yloxy)butan-2-ol), propranolol and atenolol (Baker *et al.*, 2003). In CRE reporter gene assay, the β_2AR antagonist affinity even depended on the agonist used for the stimulation of the hβ_2AR. On the contrary, using similar experimental set-up (one difference was 30 min incubation time in cAMP accumulation assay instead of 10 min) no difference in H_2R antagonist affinities was found between the test systems and the affinity was independent of the H_2R agonist used (Baker, 2008). Noteworthy, also antagonists of β_1- and β_3-adrenergic receptors bind to the β_1AR and the β_3AR, respectively, with different affinity, indicating the existence of at least two distinct conformations of each receptor (Baker, 2008).

In contrast to aforementioned results with respect of the hH_2R on CHO cells substantial differences in pK_B values were determined for H_2R antagonists comparing the $O_2^{\bullet-}$ assay (incubation time 30 min) and the cAMP assay (incubation time 10 min) on neutrophil granulocytes (Fig. 3.12). Additionally, pK_B values determined on neutrophils do not correlate well with pK_B values for the named antagonists in recombinant test system, taken as a comparison (Fig. 3.12). Since HA was used as an agonist for hH_2R stimulation in all three test systems the influence of differing agonists on pK_B values of antagonists reported by Baker and coworkers (Baker *et al.*, 2003) can be excluded. Like other GPCRs the H_2R can be phosphorylated, desensitized and internalized upon incubation with an agonist (Hill, 2006). Therefore, one could speculate that the reduced pK_B values of FAM, TIO and ZOL in the $O_2^{\bullet-}$ assay are the consequence of a larger chemical modification (phosphorylation) of the hH_2R during 30 min incubation time in the presence of the agonist HA in comparison with chemical modification during the cAMP assay, where incubation time is only 10 min. The use of a phosphorylation-deficient mutant of the β_2AR revealed that the ISO-induced

phosphorylation of the $h\beta_2AR$ is responsible for reduced affinity of the antagonist ICI 118,551 in CRE gene reporter assay (Baker *et al.*, 2003). However, it should be noted here that the incubation time in the gene reporter assay in case of the β_2AR was 10-fold longer than the incubation time for the hH_2R in the $O_2^{\bullet-}$ assay. Moreover, no divergence in hH_2R antagonist affinities was found by the same research group comparing the immediate event of cAMP accumulation and further downstream CRE gene reporter assay in recombinant test system (Baker, 2008).

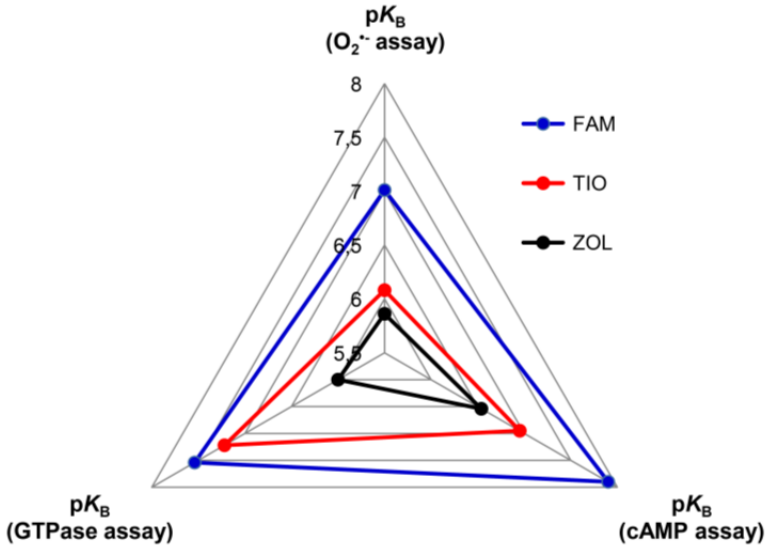

Fig. 3.12 Web of pK_B values for H_2R antagonists FAM, TIO and ZOL. On human neutrophil granulocytes, the $O_2^{\bullet-}$ assay (1 x 10^5 cells per well) was performed as described in section 3.2.3, and the cAMP assay (5 x 10^5 cells per cup) was performed as described in sections 3.2.4 and 3.2.5. $O_2^{\bullet-}$ and cAMP production were determined at submaximally effective concentration of HA (25 µM and 3 µM, respectively) in the presence of increasing concentrations of H_2R antagonists. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are from four to six independent experiments performed in triplicate. The pK_B values were calculated from the IC_{50} values according to Cheng and Prusoff (Cheng and Prusoff, 1973). Data for GTPase assay (Sf9 membranes with hH_2R-$G_{s\alpha S}$ fusion protein) were taken from Kelley *et al.* (Kelley *et al.*, 2001); the reported non-logarithmic K_B values were converted into logarithmic pK_B values.

In the history of drug research, discrepancies in the binding affinities or the pharmacological behavior of ligands played a pivotal role in the identification of new receptor subtypes. One prominent example with respect to the identification of a novel receptor subtype was the discovery of the H_2R by the late Sir James Black and his coworkers (Black *et al.*, 1972). However, on the basis of the data presented in this study the postulation of the existence of distinct H_2R subtypes on neutrophils seems unjustifiable, although the described differential effects of examined H_2R antagonists on cAMP production and $O_2^{\bullet-}$ accumulation in neutrophils are unequivocal. We could show that FAM, at a concentration of 10 µM, was

able to completely antagonize the effect of 100 µM HA, proving that the HA-induced inhibition of $O_2^{\bullet-}$ production is indeed H_2R-mediated.

Examination of β_2AR antagonists on neutrophil granulocytes to find out whether pK_B values of β_2AR antagonists also differ in the cAMP assay and the $O_2^{\bullet-}$ assay is subject of ongoing work. Moreover, studies with PKA inhibitors are planned in order to gain deeper insight into the signaling events between cAMP accumulation and $O_2^{\bullet-}$ production.

3.4.4 Conclusions

As the activation of the H_2R and the β_2AR could have beneficial anti-inflammatory effects both aminergic GPCRs were characterized on human neutrophil granulocytes, cells of the innate immune system. For comprehensive characterization the influence of the H_2R and the β_2AR on fMLP-stimulated $O_2^{\bullet-}$ production and cAMP accumulation, respectively, was assessed. Collectively, this work revealed (I) indications for functional selectivity of examined H_2R and β_2AR agonists, (II) distinct pharmacological profile of the hH_2R and the hβ_2AR on neutrophil granulocytes and (III) divergence in pK_B values for H_2R antagonist affinities between the $O_2^{\bullet-}$ assay and the cAMP assay. Further studies are needed to mechanistically explain some findings of the present study.

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Chapter 4

Interaction of N^G-acylated hetarylpropylguanidines with the N-terminus of the histamine H_2 receptor and their effects on human neutrophil granulocytes

4.1 Introduction

Histamine H_2 receptor (H_2R) antagonists such as cimetidine, ranitidine and famotidine (FAM) have been well established drugs in the therapy of gastroduodenal ulcers and gastroesophaegal reflux disease for decades (van der Goot and Timmerman, 2000). Histamine dihydrochloride in combination with interleukin-2 (Ceplene®) has come on the market very recently as an "H_2R agonist" for maintenance immunotherapy of acute myeloid leukemia in adults (Martner *et al.*, 2010). Besides the beneficial effect in acute myeloid leukemia, new H_2R agonists could be also advantageous as anti-inflammatory agents (Burde *et al.*, 1990) and positive inotropic drugs in the therapy of heart failure (Baumann *et al.*, 1984; Felix *et al.*, 1995).

N^G-acylated hetarylpropylguanidines are a new class of potent H_2R agonists (Xie *et al.*, 2006; Ghorai *et al.*, 2008; Kraus *et al.*, 2009; Birnkammer, 2011). This group of compounds was derived from guanidine-type analogues of impromidine (IMP) and arpromidine (ARP) in order to reduce basicity, improve oral bioavailability and to allow the penetration across blood-brain barrier (Xie *et al.*, 2006; Ghorai *et al.*, 2008). The most potent monovalent N^G-acylated hetarylpropylguanidines show potencies in the low two-digit nM range (Xie *et al.*, 2006; Ghorai *et al.*, 2008; Kraus *et al.*, 2009). Recently, bivalent N^G-acylated hetarylpropylguanidines were synthesized with some of them having potencies even in the one-digit nM range (Birnkammer, 2011). Whereas monovalent N^G-acylated imidazolylpropylguanidines show antagonistic and/or partial agonistic activity at the hH_1R, hH_3R and hH_4R (Xie *et al.*, 2006; Ghorai *et al.*, 2008; Igel *et al.*, 2009), N^G-acylated aminothiazolylpropylguanidines are selective partial to full hH_2R agonists (Kraus *et al.*, 2009). The same selectivity pattern was observed for bivalent N^G-acylated hetarylpropylguanidines (Birnkammer, 2011).

In general, bivalent ligands are compounds containing two pharmacophoric moieties joined by a spacer, and they are synthesized with the intention to increase affinity, potency and selectivity compared to the monovalent counterparts and to investigate receptor homo- and heterodimers (Smith and Milligan, 2010; Shonberg *et al.*, 2011). The bivalent ligand approach has been successfully applied in studies of numerous GPCRs, e.g. dopamine receptors, serotonin receptors, gonadotropin-releasing hormone receptors, opioid receptors and others (Halazy et al., 1996; Decker and Lehmann, 2007; Bonger et al., 2008; Zhang et al., 2009; Kühhorn et al., 2011; Shonberg et al., 2011). Also in our research group the bivalent ligand approach resulted in very potent agonists at the hH_2R and gpH_2R with substantially increased potency in comparison to monovalent N^G-acylated hetarylpropylguanidines (Birnkammer, 2011). Nevertheless, structure-activity relationship studies on bivalent N^G-acylated hetarylpropylguanidines revealed that spacer length of the most potent ligands is insufficient to allow simultaneous occupation of two neighboring

receptors (Portoghese, 2001; Birnkammer, 2011). It is, therefore, conceivable that the second pharmacophoric moiety binds to an additional (allosteric) binding site on the same receptor.

Characteristic of monovalent and bivalent N^G-acylated hetarylpropylguanidines is also enhanced potency and efficacy at the guinea pig (gp) H_2R compared to the human (h) H_2R ortholog as determined in GTPase assay using membrane preparations of Sf9 insect cells expressing gpH_2R-$G_{s\alpha S}$ and hH_2R-$G_{s\alpha S}$ (Xie *et al.*, 2006; Ghorai *et al.*, 2008; Kraus *et al.*, 2009; Birnkammer, 2011). As shown in Fig. 4.1, hH_2R and gpH_2R possess very high overall sequence identity of 86 %, even higher identity within transmembrane domains (96 %), but very low homology in the N-terminal sequence (66 %). Therefore, using mutagenesis study, we assessed the contribution of the N-terminus to different behavior of N^G-acylated hetarylpropylguanidines at the hH_2R and gpH_2R as well as the potential role of this receptor domain as an accessory binding site of bivalent H_2R agonists.

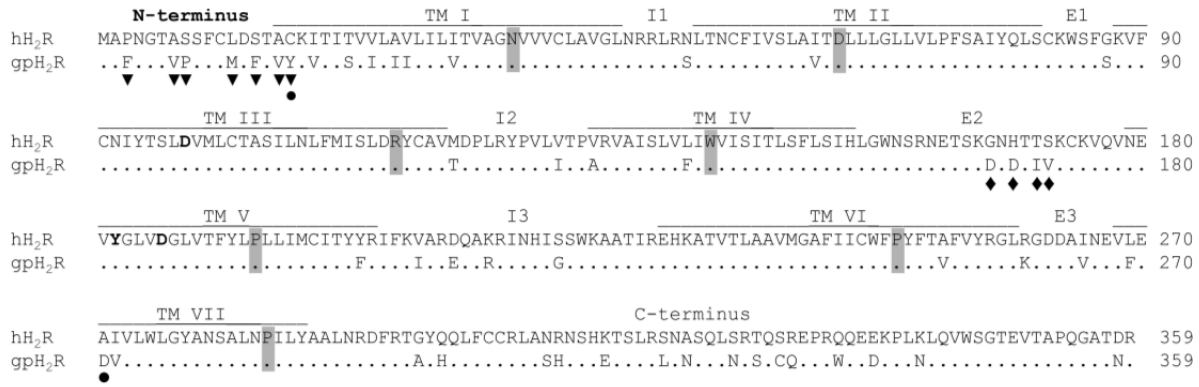


Fig. 4.1 Comparison of the amino acid sequences of the hH_2R and the gpH_2R. Amino acid sequences of the wild-type hH_2R (Gantz *et al.*, 1991) and the wild-type gpH_2R (Traiffort *et al.*, 1995) are given in one-letter code. TM I-VII, seven transmembrane domains; I1-3, first, second and third intracellular loop; E1-3, first, second and third extracellular loop. Dots in the sequence of the gpH_2R indicate identical amino acids with the hH_2R. Amino acids with grey shading represent the most conserved amino acids among aminergic G protein-coupled receptors (Shi and Javitch, 2002) and amino acids in bold indicate interaction sites of HA with the H_2R (Gantz *et al.*, 1992; Nederkoorn *et al.*, 1996). Amino acids marked with downward-facing triangle (▼) at the hH_2R were replaced by the corresponding amino acids of the gpH_2R by the generation of chimeric receptor $h_{gpNT}H_2R$ in this study. Amino acids marked with circle (●) were reported to determine the species-selectivity of monovalent guanidines and N^G-acylguanidines (Kelley *et al.*, 2001; Preuss *et al.*, 2007a) whereas amino acids marked with diamond (♦) do not influence the species-selectivity of the same class of ligands (Preuss *et al.*, 2007b).

Moreover, regarding the hH_2R, the ligands of interest were so far only characterized in recombinant test systems, namely the hH_2R-$G_{s\alpha S}$ fusion protein in Sf9 cell membranes. To make one step forward, a set of representative N^G-acylated hetarylpropylguanidines was additionally characterized on isolated human neutrophil granulocytes. Neutrophil granulocytes endogenously express H_2Rs and were therefore used as a test system for

characterization of H_2R ligands in the past (Burde *et al.*, 1990). The H_2R agonistic activity of acylguanidines was assessed by monitoring the inhibition of N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)-stimulated $O_2^{\bullet-}$ production and cyclic adenosine 3',5'-monophosphate (cAMP) accumulation in neutrophils. The signaling pathway of the fMLP-induced $O_2^{\bullet-}$ production and the interference of the latter with the signaling pathway of the H_2R in human neutrophil granulocytes are described in section 3.1.

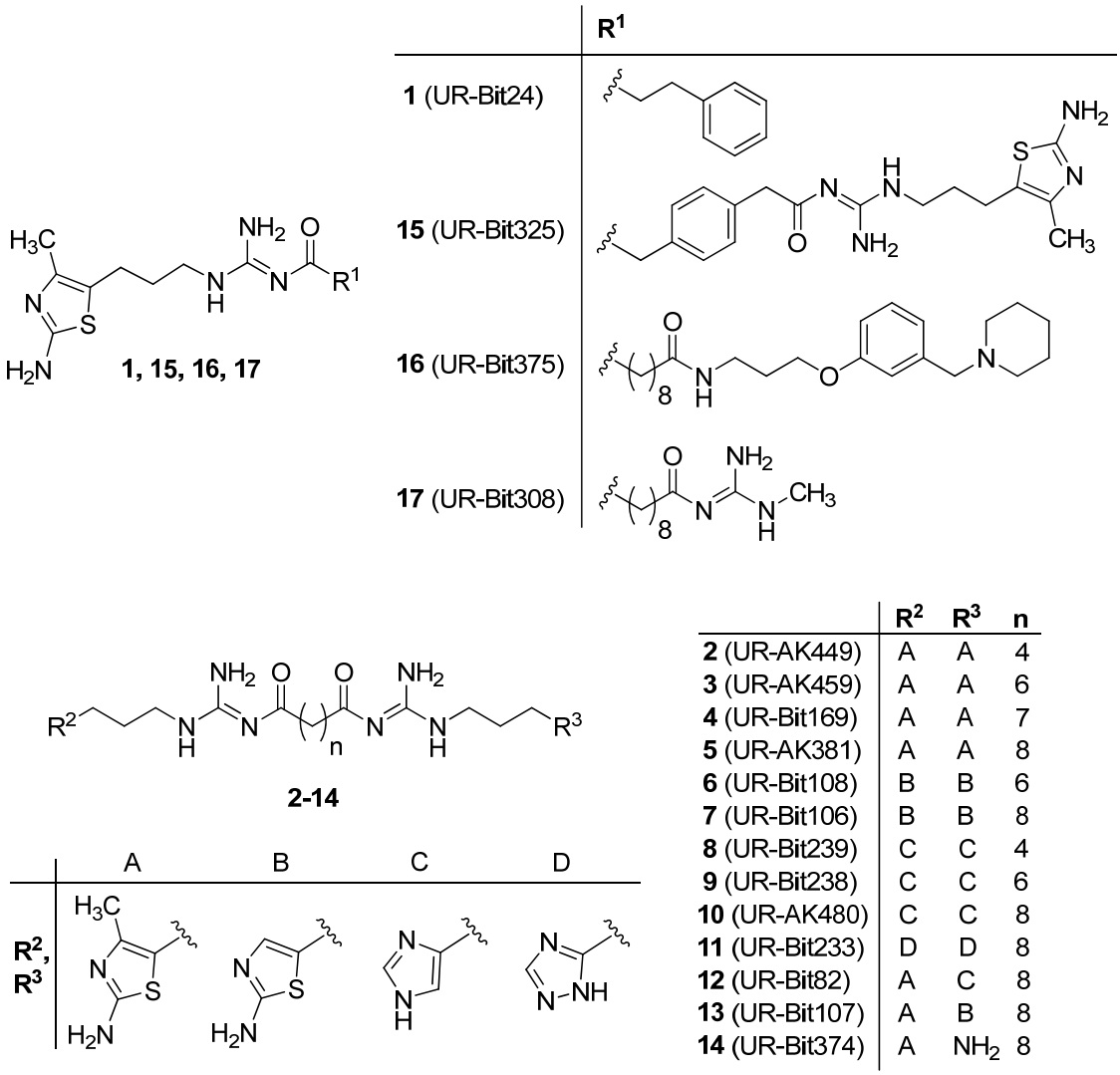
4.2 Materials and Methods

4.2.1 Materials

Histamine (HA) and thioperamide (THIO) were obtained from Tocris Bioscience (Avonmouth, Bristol, UK). Mepyramine (MEP) and FAM were from Sigma-Aldrich Chemie (Steinheim, Germany). Compound **1** (Kraus *et al.*, 2009) and ligands **2-17** (Birnkammer, 2011) were synthesized in our laboratory as described. The structures of the synthesized compounds **2-17** were confirmed by 1H NMR, 13C-NMR and mass spectroscopy spectra (HR-MS). Purity of compounds was ≥ 95 % as determined by analytical HPLC (compound purities were calculated as the percentage peak area of the analyzed compound by UV detection at 210 nm). Chemical structures of ligands **1-17** are depicted in Fig. 4.2 and an overview of potencies and efficacies, determined in recombinant test system, is given in Table 4.1. Stock solutions of HA, MEP, THIO and compound **1** (10 mM each) were prepared in Millipore water. 2 mM stock solution of FAM was dissolved in 2 mM HCl. 2 mM stock solutions of **2-17** were prepared in 20 % dimethyl sulfoxide (DMSO) (v/v). For GTPase assay, dilution series of HA and ligands **1**, **5**, **6**, **10**, **11**, **12** and **17** were prepared in water/DMSO mixture in defined ratio to achieve constant final concentration of 1 % DMSO (v/v) in test tubes. For the $O_2^{\bullet-}$ assay and the cAMP assay, all ligands were diluted with Millipore water; final concentration of DMSO in experiments was maximally 0.01 % (v/v).

The generation of pGEM-3Z-SF-hH_2R-His_6-G_{saS} has been described previously (Kelley *et al.*, 2001). DNA primers for PCR were purchased by MWG Biotech (Ebersberg, Germany). Phusion high-fidelity DNA polymerase was obtained from Finnzymes (Espoo, Finland). Restriction enzymes and T4-DNA ligase were from New England Biolabs (Ipswich, MA, USA). Insect Xpress medium was from Lonza (Walkersville, MD, USA), fetal calf serum from Biochrom (Berlin, Germany) and gentamicin from Cambrex Bio Science (Walkersville, MD, USA). BaculoGOLD transfection kit was purchased from BD PharMingen (San Diego, CA, USA). EDTA and leupeptin were from Merck (Darmstadt, Germany). Adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), adenosine 5'-[β,γ-imido]triphosphate and creatine kinase were purchased from Roche (Mannheim, Germany). Benzamidine was obtained from Acros Organics (Geel, Belgium). Phenylmethylsulfonyl fluoride, creatine

phosphate and bovine serum albumin were from Sigma-Aldrich Chemie (Steinheim, Germany)). [γ-^{33}P]GTP was synthesized using guanosine 5'-diphosphate (GDP) and [γ-^{33}P]P_i (3,000 Ci/mmol orthophosphoric acid) by analogy with the synthesis of [γ-^{32}P]GTP described before (Walseth and Johnson, 1979). GDP was purchased from Sigma-Aldrich Chemie (Steinheim, Germany) and [γ-^{33}P]P_i from Hartmann Analytic (Braunschweig, Germany).



	R^2	R^3	n
2 (UR-AK449)	A	A	4
3 (UR-AK459)	A	A	6
4 (UR-Bit169)	A	A	7
5 (UR-AK381)	A	A	8
6 (UR-Bit108)	B	B	6
7 (UR-Bit106)	B	B	8
8 (UR-Bit239)	C	C	4
9 (UR-Bit238)	C	C	6
10 (UR-AK480)	C	C	8
11 (UR-Bit233)	D	D	8
12 (UR-Bit82)	A	C	8
13 (UR-Bit107)	A	B	8
14 (UR-Bit374)	A	NH_2	8

Fig. 4.2 Structures of the examined N^G-acylated hetarylpropylguanidine type histamine H_2 receptor agonists. Laboratory code for each ligand is given in parenthesis.

10 x Dulbecco's phosphate buffered saline (DPBS) without Ca^{2+} and Mg^{2+} (pH 6.5 – 7.0) was purchased from PAN Biotech (Aidenbach, Germany) and Biocoll separating solution from Biochrom (Berlin, Germany). Trypan blue solution, ferricytochrome c, cytochalasin B, fMLP and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma-Aldrich Chemie (Steinheim, Germany). Solvents for extraction and HPLC analysis were purchased as follows: HPLC-gradient grade water and methanol from J. T. Baker (Deventer, The Netherlands),

ammonium acetate from Sigma-Aldrich Chemie (Steinheim, Germany) and acetic acid from Riedel-de Haen (Hannover-Seelze, Germany). cAMP (> 99 %) was obtained from Biolog Life Science Institute (Bremen, Germany) and tenofovir from the National Institute of Health (Bethesda, MD, USA). Morphological investigation of neutrophil granulocytes was performed with Zeiss microscope Axiovert 200M (Göttingen, Germany).

4.2.2 Construction of the cDNA for the $h_{gpNT}H_2R$-$G_{s\alpha S}$ fusion protein

The cDNA for $h_{gpNT}H_2R$-$G_{s\alpha S}$ was generated by sequential overlap-extension PCR, using pGEM-3Z-SF-hH_2R-His_6-G_{saS} as template, by analogy with a previously described approach (Preuss *et al.*, 2007b). In PCR 1A, the DNA region encoding the cleavable signal peptide from influenza hemagglutinin (S), the FLAG epitope (F) recognized by the M1 monoclonal antibody and the N-terminus of gpH_2R was amplified. The sense primer annealed with 27 bp of pGEM-3Z in front of the 5'-end of SF. The antisense primer with the sequence 5'-***T***AT***A***CGGTA***A***AGTCCA***T***GCAAAAGGAAG***G***G***A***CTGTGCCATTG***AA***TGCCAT GGCGTCATCATCGTC-3' was used to generate Pro3→Phe3, Ala7→Val7, Ser8→Pro8, Leu12→Met12, Ser14→Phe14, Ala16→Val16 and Cys17→Try17 mutations in the region of N-terminus and a new *Bst* Z17I restriction site (GTATAC). Amino acids at positions 16 (Ala) and 17 (Cys) in the hH_2R are, according to Kelley and colleagues (Kelley *et al.*, 2001), located in TM I (Fig. 4.1), but were included in the mutagenesis study because of the close proximity to the N-terminus. In PCR 1B, the DNA sequence of guinea pig N-terminus, the rest of hH_2R, hexahistidine tag (His_6) and G_{saS} was amplified. The sense primer encoded the sequence 5'-***TT***CAATGGCACAG***T***C***C***CTTCCTTTTGC***A***TGGACT***T***TACCG***T***AT***A***CAAGAT CACCATCACCGTGGTCC-3' to generate Pro3→Phe3, Ala7→Val7, Ser8→Pro8, Leu12→Met12, Ser14→Phe14, Ala16→Val16 and Cys17→Try17 mutations in the region of N-terminus and a new *Bst* Z17I restriction site. The antisense primer annealed with 21 bp of pGEM-3Z behind the stop codon and downstream to *Xba* I site. In PCR 2, using the sense primer of PCR 1A and the antisense primer of PCR 1B, the products of PCR 1A and 1B annealed in the region of newly created canine N-terminus and a new *Bst* Z17I restriction site. The product of PCR 2 encoded the complete cDNA for SF-$h_{gpNT}H_2R$-His_6-$G_{s\alpha S}$ protein. This product was double-digested with *Sac* I and *Xba* I and cloned into the pGEM-3Z-SF-hH_2R-His_6-G_{saS} plasmid digested with the same enzymes. The pGEM-3Z-SF-$h_{gpNT}H_2R$-His_6-G_{saS} DNA was digested with *Sac* I and *Xba* I and cloned into the baculovirus transfer vector pVL1392-SF-hH_2R-His_6-G_{saS} digested with the same enzymes. The sequence of SF-$h_{gpNT}H_2R$-His_6-G_{saS}, cloned into the pGEM-3Z plasmid, was verified by restriction enzyme analysis and sequencing (Entelechon, Regensburg, Germany).

4.2.3 Sf9 insect cell culture, generation of recombinant baculoviruses and membrane preparation

Sf9 insect cell culture and generation of recombinant baculoviruses have been described recently (Schneider *et al.*, 2009; Schnell *et al.*, 2010). Sf9 cells were cultured in 250- or 500-ml disposable Erlenmeyer flasks at 28 °C under rotation at 150 rpm in Insect Xpress medium supplemented with 5 % (v/v) fetal calf serum and 0.1 mg/ml gentamicin. Cells were maintained at a density of 0.5 – 6.0 x 10^6 cells/ml. Recombinant baculoviruses encoding the $h_{gpNT}H_2R\text{-}G_{s\alpha S}$ were generated in Sf9 insect cells using the BaculoGOLD transfection kit according to manufacturer's instructions. After initial transfection, high-titer virus stocks were generated by two sequential virus amplifications. In the first amplification, cells were seeded at 2.0 x 10^6 cells/ml and infected with a 1:100 dilution of the supernatant fluid from the initial transfection. Cells were cultured for 7 days, resulting in death of virtually the entire cell population. The supernatant of this infection was harvested and stored under light protection at 4 °C. In a second amplification, cells were seeded at 3.0 x 10^6 cells/ml and infected with a 1:20 dilution of the supernatant fluid from the first amplification. Cells were cultured for 48 hours and afterwards, the supernatant fluid was harvested. After the 48 hours culture period, the most of cells showed signs of infection (altered morphology), but the majority of cells was still intact. The supernatant fluid from the second amplification was stored under light protection at 4 °C and used as virus stock for membrane preparation.

Before infection, Sf9 cells were sedimented by centrifugation and suspended in fresh medium. Cells were seeded at a density of 3.0 x 10^6 cells/ml and infected with high-titer baculovirus stock encoding $h_{gpNT}H_2R\text{-}G_{s\alpha S}$ (1:100 dilution). Cells were cultured for 48 h at 28 °C under rotation at 150 rpm before the membrane preparation was performed as described before (Seifert *et al.*, 1998). All steps during membrane preparation were performed at 4 °C. After 48 hours culture period, cells were washed by centrifugation step at 170 x g for 10 min, discarding the supernatant and resuspending the cell pellet in 50 ml of PBS buffer (137 mM NaCl, 2.6 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 1.5 mM KH_2PO_4 and 0.8 mM Na_2HPO_4, pH 7.4). After repeating the centrifugation step, the supernatant was removed and the pellet suspended in 15 ml of lysis buffer, containing 1 mM EDTA, 10 mM Tris/HCl pH 7.4, 0.2 mM phenylmethylsulfonyl fluoride, 10 µg/ml benzamidine and 10 µg/ml leupeptin, and homogenized in a Dounce homogenizer with 25 strokes. After centrifugation at 40 x g for 5 min, the supernatant contained the membranes and the pellet nuclei and unbroken cells. The supernatant was then transferred to a plastic tube and centrifuged at 38,500 x g for 20 min. The pellet containing the membranes was resuspended in 20 ml of lysis buffer and centrifuged once again at 38,500 x g for 20 min. The membrane pellet was then suspended in 25 ml of binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4) and

homogenized by a syringe with 20 strokes. Aliquots of membranes (1 ml) were stored at -80 °C until use.

4.2.4 Steady-state GTPase activity assay

Steady-state GTPase activity assays, using [γ-^{33}P]GTP as radioligand, were essentially conducted as described (Preuss *et al.*, 2007b). The membranes were thawed, sedimented by centrifugation (18,000 x g, 10 min, 4°C) and resuspended in 10 mM Tris/HCl, ph 7.4. Assay tubes contained membranes expressing $h_{gpNT}H_2R$-$G_{s\alpha S}$ fusion protein (5-10 µg of protein per tube), 1.0 mM $MgCl_2$ (final concentration after the addition of [γ-^{33}P]GTP), 100 µM EDTA, 100 µM ATP, 100 nM GTP, 100 µM adenosine 5'-[β,γ-imido]triphosphate, 1.2 mM creatine phosphate, 1 µg creatine kinase, 0.2 % (w/v) bovine serum albumin in 50 mM Tris/HCl, pH 7.4 and ligands at investigated concentrations. Reaction mixtures (80 µl) were incubated for 2 min at 25 °C before the addition of 20 µl of [^{33}P]GTP (0.05 µCi per tube). Reactions were conducted for 20 min at 25°C and terminated by the addition of 900 µl of slurry consisting of 5 % (w/v) activated carcoal (absorbs nucleotides, but not P_i) and 50 mM NaH_2PO_4, pH 2.0. Reaction mixtures were then centrifuged at room temperature (7 min, 15,000 x g). Six hundert µl of resulting supernatant was removed and $^{33}P_i$ was determined by liquid scintillation counting using OptiPhase Supermix Cocktail (PerkinElmer, Groningen, The Netherlands). Enzyme activities were corrected for spontaneous degradation of [γ-^{33}P]GTP, which was determined in tubes containing all of the above described components and additionally a high concentration of unlabeled GTP (1 mM) that, by competition with [γ-^{33}P]GTP, prevents [γ-^{33}P]GTP hydrolysis by enzymatic activities present in Sf9 membranes. Spontaneous [γ-^{33}P]GTP degradation normally amounted to < 1 % of the total amount of radioactivity added. The experimental conditions ensured that no more than 16 % of the total amount of [γ-^{33}P]GTP added were converted to $^{33}P_i$.

4.2.5 Isolation of human neutrophils

See section 3.2.2.

4.2.6 Superoxide anion generation ($O_2^{\bullet-}$ assay)

See section 3.2.3.

4.2.7 cAMP accumulation and extraction from neutrophils (cAMP assay)

See section 3.2.4.

4.2.8 Quantitation of cAMP by HPLC-MS/MS

See section 3.2.5.

4.2.9 Miscellaneous

Protein concentrations were determined using the Bio-Rad DC protein assay kit (Hercules, CA). Chromatograms, obtained during the HPLC-MS/MS analysis, were analyzed with the Analyst Software 1.5.1 (AB Sciex, Foster City, CA). Data from GTPase assay, $O_2^{\bullet-}$ assay and cAMP assay were analyzed with the Prism 5.01 software (GraphPad, San Diego, CA). The means ± S.E.M. were always determined by the analysis of at least three independent experiments, performed in duplicate or triplicate, if not indicated otherwise. pEC_{50} and E_{max} values in Table 4.1, respectively, were statistically analyzed using one-way ANOVA, followed by Bonferroni's multiple comparison test. Statistical significance was defined as $p < 0.05$ (95 % confidence interval).

4.3 Results

4.3.1 Characterization of HA and N^G-acylated hetarylpropylguanidines at the $h_{gpNT}H_2R\text{-}G_{s\alpha S}$ in steady-state GTPase assay

In order to assess the potential involvement of the N-terminus in different characteristics of monovalent as well as bivalent N^G-acylated hetarylpropylguanidines at $hH_2R\text{-}G_{s\alpha S}$ and $gpH_2R\text{-}G_{s\alpha S}$ (Kraus *et al.*, 2009; Birnkammer, 2011), the chimeric fusion receptor $h_{gpNT}H_2R\text{-}G_{s\alpha S}$ was constructed by overlap-extension PCR, in which the N-terminal amino acid sequence of the hH_2R was exchanged by the counterpart sequence of the gpH_2R (Fig. 4.1). Afterwards, Sf9 membranes, expressing $h_{gpNT}H_2R\text{-}G_{s\alpha S}$ fusion protein, were characterized with the series of seven N^G-acylated hetarylpropylguanidines (compounds **1**, **5**, **6**, **10**, **11**, **12** and **17**) and with the standard agonist HA in the steady-state GTPase assay. For $h_{gpNT}H_2R\text{-}G_{s\alpha S}$, basal GTPase activities ranged from 3.3 to 3.8 pmol/mg/min and GTPase activity after stimulation with 100 µM HA from 9.0 to 11.4 pmol/mg/min.

Potencies and efficacies of the examined ligands at the $h_{gpNT}H_2R\text{-}G_{s\alpha S}$ as well as comparison with potencies and efficacies at the $hH_2R\text{-}G_{s\alpha S}$ and the $gpH_2R\text{-}G_{s\alpha S}$ are shown in Table 4.1. For HA, **10**, **12** and **17**, statistical analysis of the data revealed a difference neither in potency nor in efficacy, when the mutant receptor $h_{gpNT}H_2R\text{-}G_{s\alpha S}$ was compared with the $hH_2R\text{-}G_{s\alpha S}$. Interestingly, the pEC_{50} value of the monovalent (2-amino-4-methylthiazolyl)propylguanidine **1** and the E_{max} value of the symmetrical bivalent (2-aminothiazolyl)propylguanidine **6** at the $h_{gpNT}H_2R\text{-}G_{s\alpha S}$ were significantly lower than the respective pEC_{50} and E_{max} values at the $hH_2R\text{-}G_{s\alpha S}$. On the contrary, in case of the

symmetrical bivalent triazolylpropylguanidine **11**, the pEC_{50} and E_{max} of at the $hH_2R\text{-}G_{s\alpha S}$ tend to those determined at the $gpH_2R\text{-}G_{s\alpha S}$. The E_{max} value of the symmetrical bivalent (2-amino-4-methylthiazolyl)propylguanidine **5** even reaches the same efficacy as at the $gpH_2R\text{-}G_{s\alpha S}$.

Table 4.1 Potencies and efficacies of HA and N^G-acylated hetarylpropylguanidines on $hH_2R\text{-}G_{s\alpha S}$, $gpH_2R\text{-}G_{s\alpha S}$ and $h_{gpNT}H_2R\text{-}G_{s\alpha S}$ fusion proteins, determined in steady-state GTPase assay.

Cpd.	$hH_2R\text{-}G_{s\alpha S}$[a]		$gpH_2R\text{-}G_{s\alpha S}$[a]		$h_{gpNT}H_2R\text{-}G_{s\alpha S}$[b]	
	pEC_{50}	E_{max}	pEC_{50} (pK_B)	E_{max}	pEC_{50}	E_{max}
HA[c]	6.01 ± 0.02	1.00	6.10 ± 0.08	1.00	5.93 ± 0.04	1.00
1[c]	7.68 ± 0.08	0.79 ± 0.01	8.13 ± 0.03**	0.76 ± 0.01	7.31 ± 0.03$^{*,+++}$	0.82 ± 0.01^{+}
2	7.24 ± 0.22	0.68 ± 0.03	8.56 ± 0.30	0.90 ± 0.01	n.d.	n.d.
3	7.30 ± 0.25	0.62 ± 0.03	9.20 ± 0.16	0.81 ± 0.03	n.d.	n.d.
4	7.45 ± 0.15	0.43 ± 0.04	8.57 ± 0.16	0.92 ± 0.04	n.d.	n.d.
5[c]	8.11 ± 0.25	0.53 ± 0.04	9.41 ± 0.14**	0.74 ± 0.05*	7.94 ± 0.05^{++}	0.73 ± 0.01*
6[c]	7.53 ± 0.02	0.81 ± 0.01	8.87 ± 0.28***	1.00 ± 0.03***	7.40 ± 0.04^{+++}	0.73 ± 0.02$^{*,+++}$
7	7.67 ± 0.07	0.76 ± 0.03	8.30 ± 0.22	0.94 ± 0.01	n.d.	n.d.
8	6.67 ± 0.34	0.68 ± 0.04	7.98 ± 0.11	1.01 ± 0.02	n.d.	n.d.
9	7.36 ± 0.22	0.77 ± 0.12	8.49 ± 0.34	1.18 ± 0.01	n.d.	n.d.
10[c]	8.18 ± 0.04	0.88 ± 0.05	8.93 ± 0.06***	1.05 ± 0.05*	8.10 ± 0.03^{+++}	0.87 ± 0.01
11[c]	6.82 ± 0.05	0.48 ± 0.02	7.97 ± 0.02***	0.96 ± 0.05***	7.10 ± 0.03$^{*,+++}$	0.62 ± 0.02$^{*,++}$
12[c]	8.13 ± 0.04	0.79 ± 0.06	9.33 ± 0.12***	1.03 ± 0.04*	8.14 ± 0.06^{+++}	0.79 ± 0.01^{+}
13	7.85 ± 0.16	0.79 ± 0.03	8.47 ± 0.30	0.89 ± 0.04	n.d.	n.d.
14	7.68 ± 0.11	0.58 ± 0.03	8.15 ± 0.10	0.94 ± 0.04	n.d.	n.d.
15	6.63 ± 0.23	0.74 ± 0.03	7.86 ± 0.06	0.83 ± 0.06	n.d.	n.d.
16	7.32 ± 0.06	0.60 ± 0.03	*(7.91 ± 0.02)*		n.d.	n.d.
17[c]	7.84 ± 0.09	0.60 ± 0.03	8.70 ± 0.05***	0.89 ± 0.04***	7.81 ± 0.05^{+++}	0.60 ± 0.01^{+++}

[a] pEC_{50} (pK_B) and E_{max} values at $hH_2R\text{-}G_{s\alpha S}$ and $gpH_2R\text{-}G_{s\alpha S}$ were taken from Preuss *et al.* (2007b) for HA, from Kraus *et al.* (2009) for compound **1** and from Birnkammer (2011) for compounds **2-17**.

[b] GTPase activity in Sf9 membranes (5 to 10 µg protein per tube), expressing $h_{gpNT}H_2R\text{-}G_{s\alpha S}$ fusion protein, was determined as described in section 4.2.4. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are the means ± S.E.M. of three independent experiments performed in duplicate. The efficacy (E_{max}) of HA was set to 1.00 and the efficacies of other ligands were referred to this value.

[c] pEC_{50} and E_{max} values were compared using one-way ANOVA, followed by Bonferroni`s multiple comparison test (pEC_{50} or E_{max} significantly different to: $^{*}hH_2R\text{-}G_{s\alpha S}$, $^{+}gpH_2R\text{-}G_{s\alpha S}$; one symbol: $p < 0.05$, two symbols: $p < 0.01$, three symbols: $p < 0.001$).

n.d. Not determined.

4.3.2 Characterization of HA and N^G-acylated hetarylpropylguanidines on neutrophil granulocytes in the $O_2^{\bullet-}$ assay

We aimed at characterizing the N^G-acylated hetarylpropylguanidine group of H_2R ligands in a physiologically relevant test system. Neutrophil granulocytes express H_2Rs and sufficient amount of neutrophils can be isolated from human blood effectively. Therefore, we choose these white blood cells for the verification of the very potent agonistic activity of N^G-acylated hetarylpropylguanidine, determined for the hH_2R in recombinant test system. Superoxide anion production, stimulated by fMLP *via* formyl peptide receptor on neutrophil granulocytes, is inhibited by elevated levels of cAMP in cells (Burde *et al.*, 1989; Burde *et al.*, 1990). The H_2R is G_s-coupled receptor and that is why the agonistic activity of examined compounds at the H_2R can be monitored as an inhibition of fMLP-stimulated $O_2^{\bullet-}$ production (Fig. 4.3, Table 4.2).

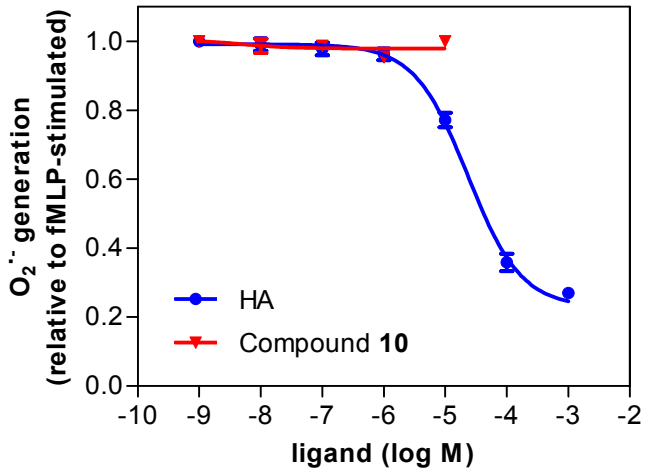


Fig. 4.3 Concentration-response curves for HA and compound 10 in the $O_2^{\bullet-}$ assay. $O_2^{\bullet-}$ production in human neutrophil granulocytes (1 x 10^5 cells per well) was monitored by measurement of superoxide dismutase-inhibitable reduction of ferricytochrome c at 550 nm as described in section 3.2.3. Data shown are from three (compound **10**) and eight (HA) independent experiments, performed in triplicate (data points are means ± S.E.M.). Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves.

Table 4.2 Comparison of potencies and efficacies of HA and selected N^G-acylated hetarylpropylguanidines in different test systems.

Compound	$O_2^{\bullet-}$ assay (hH_2R on neutrophil granulocytes)		cAMP assay (hH_2R on neutrophil granulocytes)		GTPase assay (recombinant protein hH_2R-$G_{s\alpha S}$)	
	pIC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}
HA	4.65 ± 0.06	1.00	5.47 ± 0.03	1.00	6.01 ± 0.02[b]	1.00
1	4.16 ± 0.11	0.86 ± 0.02[a]	7.08 ± 0.20	0.33 ± 0.03	7.65[c]	0.78 ± 0.02[c]
5	no effect		7.28 ± 0.20	0.25 ± 0.02	8.11 ± 0.25[d]	0.53 ± 0.04[d]
10	no effect		7.36 ± 0.12	0.31 ± 0.02	8.21 ± 0.07[d]	0.81 ± 0.02[d]
12	no effect		7.55 ± 0.21	0.26 ± 0.03	8.12 ± 0.04[d]	0.76 ± 0.05[d]

On human neutrophil granulocytes, the $O_2^{\bullet-}$ assay (1 x 10^5 cells per sample) was performed as described in section 3.2.3 and cAMP assay (5 x 10^5 cells per cup) was performed as described in sections 3.2.4 and 3.2.5. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are the means ± S.E.M. of three to eight independent experiments performed in triplicate. The efficacy (E_{max}) of HA was set to 1.00 and the efficacies of other ligands were referred to this value.

[a] Up to 100 µM, concentration-response curve for compound **1** did not achieve saturation, therefore inhibition of fMLP-stimulated $O_2^{\bullet-}$ production at fixed concentration 100 µM (relative to HA) is listed.

[b] Data were taken from Preuss *et al.* (2007b).

[c] Data were taken from Kraus *et al.* (2009).

[d] Data were taken from Birnkammer (2011).

Both, HA (100 µM) and the monovalent (2-amino-4-methylthiazolyl)propylguanidine **1** (100 µM), reduced the fMLP-stimulated $O_2^{\bullet-}$ production by 66 % on average (Table 4.3). A drastic decrease in potency was observed for both ligands compared to the data from GTPase assay (Table 4.2). No significant inhibition of the fMLP-stimulated $O_2^{\bullet-}$ production was detected in case of bivalent ligands **2-16** at a concentration of 1 µM (Table 4.3). The bivalent ligands were screened for their effect at 1 µM because of two reasons. Firstly, in steady-state GTPase assay, these ligands showed ~4.3-130-fold higher potency than HA at the hH_2R-$G_{s\alpha S}$. And secondly, bivalent ligands of acylguanidine type are cytotoxic at higher concentrations (see also section 4.4.2). Some bivalent ligands (**10**, **12**) were tested at concentrations up to 500 µM (data not shown). At higher concentrations (100 µM, 500 µM), $O_2^{\bullet-}$ production in neutrophil granulocytes was reduced; this did not happen *via* specific inhibition of $O_2^{\bullet-}$ production but was due to cell death. This was evident from the morphological examination of neutrophil granulocytes (Fig. 4.4).

Table 4.3 Effects of HA and N^G-acylated hetarylpropylguanidines 1-16 on fMLP-stimulated $O_2^{\bullet-}$ production in neutrophil granulocytes.

Compound	$O_2^{\bullet-}$ production (relative to fMLP-stimulated $O_2^{\bullet-}$ production)	Compound	$O_2^{\bullet-}$ production (relative to fMLP-stimulated $O_2^{\bullet-}$ production)
HA	0.34 ± 0.03		
1	0.34 ± 0.03	**9**	0.92 ± 0.01
2	0.97 ± 0.04	**10**	0.95 ± 0.01
3	0.99 ± 0.03	**11**	0.98 ± 0.01
4	1.00 ± 0.04	**12**	0.96 ± 0.01
5	0.97 ± 0.03	**13**	0.97 ± 0.01
6	1.00 ± 0.07	**14**	0.84 ± 0.15
7	0.97 ± 0.02	**15**	0.99 ± 0.01
8	0.98 ± 0.01	**16**	1.01 ± 0.01

$O_2^{\bullet-}$ production was monitored in human neutrophil granulocytes (1 x 10^5 cells per well) as described in section 3.2.3. Data shown are the means ± S.E.M. of three independent experiments performed in triplicate, except in case of HA, where 25 independent experiments in triplicate were conducted. The fMLP-stimulated $O_2^{\bullet-}$ production was set to 1.00 and the $O_2^{\bullet-}$ production in the presence of examined compounds was referred to this value. Inhibition of fMLP-stimulated $O_2^{\bullet-}$ production was measured at a fixed concentration of 100 µM for HA or compound **1** and at a fixed concentration of 1 µM for compounds **2-16**.

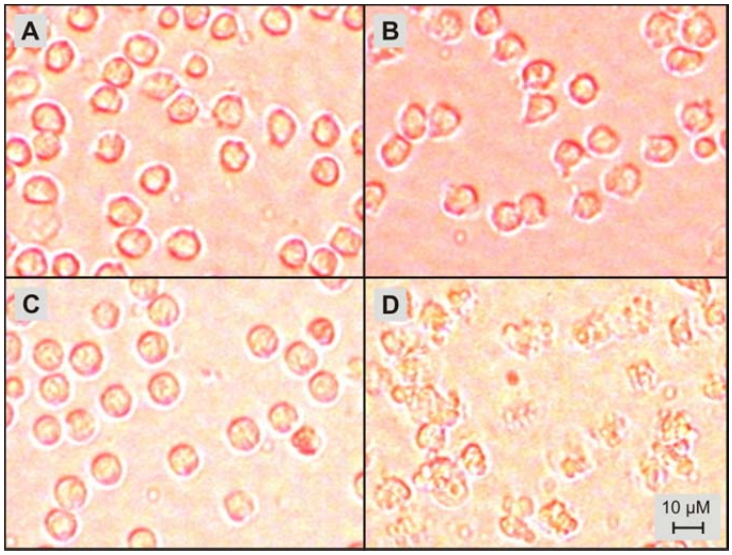


Fig. 4.4 Morphology of neutrophil granulocytes after the performance of the $O_2^{\bullet-}$ assay. The $O_2^{\bullet-}$ assay in human neutrophil granulocytes (1 x 10^5 cells per well) was performed as described in section 3.2.3. Cells were imaged approximately 1-2 hours after begin of the $O_2^{\bullet-}$ assay. The $O_2^{\bullet-}$ assay was performed under control conditions (**A**) and in the presence of ligands at concentrations as follows: 1mM HA (**B**), 1 mM compound **1** (**C**) and 500 µM compound **12** (**D**).

The effect of 100 µM HA was completely counteracted by FAM (10 µM), but not by MEP (1 µM) and THIO (10 µM), indicating solely H_2R-mediated inhibition of $O_2^{\bullet-}$ production by the endogenous ligand HA (see section 3.3.1). Additionally, the effect of HA could be almost completely antagonized by symmetrical bivalent acylguanidine **5** (Fig. 4.5A and B). On the contrary, the effect of monovalent acylguanidine **1** could not be antagonized by 10 µM FAM (Fig. 4.5A).

It is also noteworthy that all examined ligands in our study did neither cause reduction of ferricytochrome c nor stimulation of $O_2^{\bullet-}$ production *per se* (data not shown).

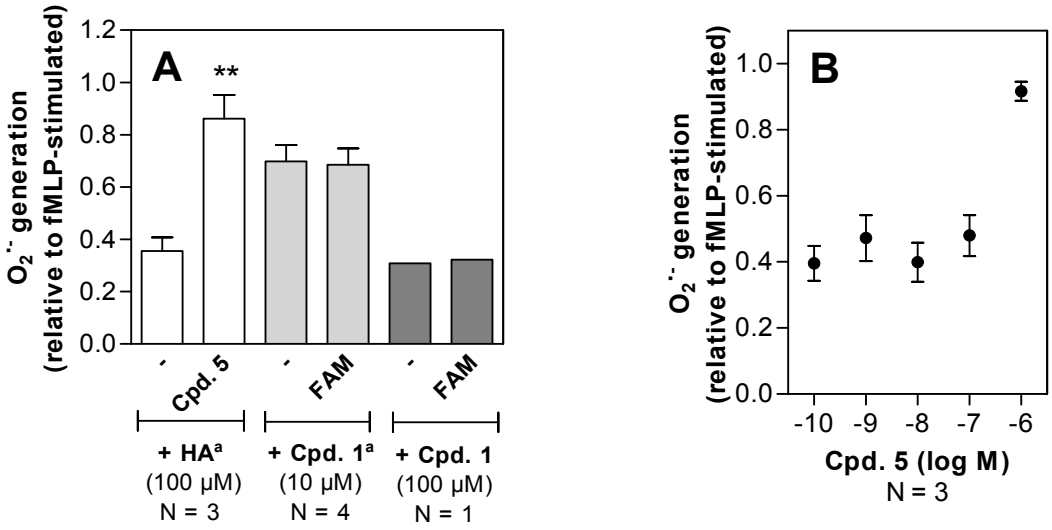


Fig. 4.5 Antagonistic effect of compound 5 and FAM on HA- or compound 1-induced inhibition of $O_2^{\bullet-}$ production. The $O_2^{\bullet-}$ production in human neutrophil granulocytes (1 x 10^5 cells per well) was monitored by measurement of ferricytochrome c reduction at 550 nm as described in section 3.2.3. Data shown are means (± S.E.M.) of one to four experiments, performed in triplicate. **(A)** Effect of compound **5** (1 µM) and FAM (10 µM) at fixed concentrations. [a] The inhibition of $O_2^{\bullet-}$ production by HA in the absence and in the presence of compound **5** and the inhibition of $O_2^{\bullet-}$ production by compound **1** (10 µM) in the absence and in the presence of FAM, respectively, were compared with the unpaired two-tailed t-test (**, $p < 0.01$). **(B)** Effect of increasing concentrations of compound **5**. $O_2^{\bullet-}$ production was determined at fixed concentration of HA (25 µM, submaximally effective concentration) in the presence of increasing concentrations of compound **5**.

4.3.3 Characterisation of HA and N^G-acylated hetarylpropylguanidines on neutrophil granulocytes in the cAMP assay

Because of the lack of H_2R agonistic activity of examined acylguanidines **2-16** in the $O_2^{\bullet-}$ assay, we decided to determine the effect of these compounds on cAMP generation in neutrophil granulocytes. Production of cAMP in cells is an event rather proximal to ligand-H_2R receptor interaction whereas monitoring of the $O_2^{\bullet-}$ production is a rather indirect way to assess agonistic activity at the H_2R (see section 3.1).

As summarized Table 4.4, N^G-acylated hetarylpropylguanidines **1-16** acted as very weak to moderate hH_2R agonists. On the basis of these screening results at fixed concentrations, we decided to investigate the effect of HA and compounds **1**, **5**, **10** and **12** in more detail. Compound **1** is a very potent monovalent N^G-acylated aminothiazolylpropylguanidine at the hH_2R-$G_{s\alpha S}$ fusion protein (Kraus *et al.*, 2009) and compounds **5**, **10** and **12** are the most potent bivalent N^G-acylated hetarylpropylguanidines in one-digit nM range, characterized at the hH_2R-$G_{s\alpha S}$ so far (Birnkammer, 2011). The potency of HA is reduced compared to the potency determined in GTPase assay, but still higher than the potency in $O_2^{\bullet-}$ assay (Table 4.2). For compounds **1**, **5**, **10** and **12**, lower potency in two-digit nM range and drastic drop in efficacy (2.3 - 2.9-fold) compared to the data in recombinant test system was observed (Table 4.2, Fig. 4.6).

Table 4.4 Effects of HA and N^G-acylated hetarylpropylguanidines 1-16 on cAMP accumulation in neutrophil granulocytes.

Compound	cAMP accumulation (relative to HA)	Compound	cAMP accumulation (relative to HA)
HA	1.00		
1	0.33 ± 0.06	**9**	0.39 ± 0.06
2	0.07 ± 0.01	**10**	0.28 ± 0.02
3	0.18 ± 0.06	**11**	0.09 ± 0.05
4	0.14 ± 0.04	**12**	0.26 ± 0.05
5	0.22 ± 0.03	**13**	0.18 ± 0.04
6	0.30 ± 0.04	**14**	0.07 ± 0.03
7	0.25 ± 0.03	**15**	0.13 ± 0.04
8	0.12 ± 0.03	**16**	0.07 ± 0.05

cAMP accumulation was monitored in neutrophil granulocytes (5×10^5 cells per sample) by HPLC-MS/MS system as described in sections 3.2.4 and 3.2.5. Data shown are the means ± S.E.M. of three to five independent experiments performed in triplicate. The cAMP production at fixed concentration of 100 µM for HA was set to 1.00 and the cAMP production at fixed concentrations of other examined compounds (10 µM for **1**; 1 µM for **2-16**) was referred to this value.

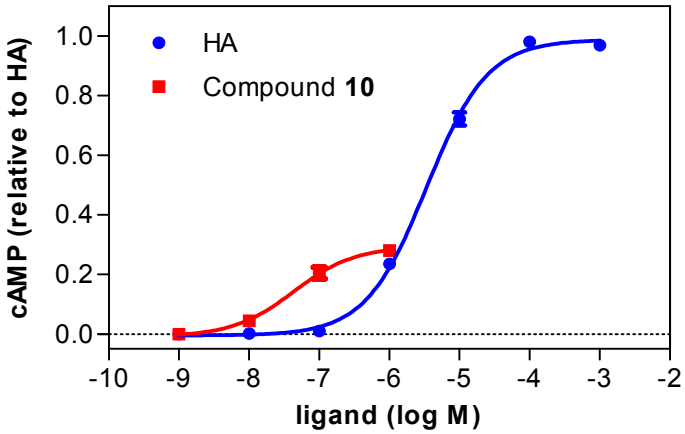


Fig. 4.6 Concentration-response curves for HA and compound 10 in the cAMP assay. cAMP concentration in human neutrophil granulocytes (5 x 10^5 cells per sample) was monitored by HPLC-MS/MS system as described in sections 3.2.4 and 3.2.5 Data shown are from three (compound **10**) and five (HA) independent experiments, performed in duplicate or triplicate (data points are mean ± S.E.M.). Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves.

In **Fig. 4.7**, the antagonistic effect of MEP, FAM and THIO on compound **1**- and compound **10**-mediated cAMP elevation in neutrophil granulocytes is shown. In case of compound **10**, cAMP level could be almost completely reduced with FAM, whereas MEP and THIO did not influence the cAMP production. In contrast to the results of the $O_2^{\bullet-}$ assay, stimulation of cAMP production by compound **1** could be at least partially reduced by FAM.

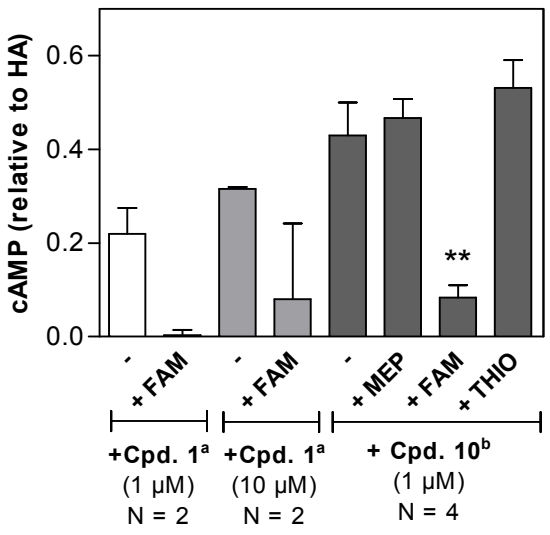


Fig. 4.7 Antagonistic effect of MEP, FAM and THIO on compound 1- and compound 10-induced cAMP accumulation in neutrophil granulocytes. cAMP accumulation was monitored in neutrophil granulocytes (5 x 10^5 cells per sample) by HPLC-MS/MS system as described in sections 3.2.4 and 3.2.5. Data shown are means of two to four experiments, performed in triplicate. MEP, mepyramine at a concentration of 1 µM; FAM, famotidine at a concentration of 10 µM; THIO, thioperamide at a concentration of 10 µM. [a] The cAMP production by 1 µM of compound **1** in the absence and in the presence of 10 µM FAM and the cAMP production by 10 µM of compound **1** in the absence and in the presence of 10 µM FAM, respectively, were compared with unpaired two-tailed t-test (no significant difference found). [b] The stimulation of cAMP production by compound **10** in the absence of antagonists (reference value) was compared with the stimulation of cAMP production by compound **10** in the presence of MEP, FAM and THIO, respectively, using one-way ANOVA, followed by Dunnett's multiple comparison test (**, $p < 0.01$).

4.4 Discussion

N^G-acylated hetarylpropylguanidines, synthesized in our laboratory, are very potent H_2R agonists as determined in GTPase and adenylate cyclase assays using membrane preparations of Sf9 insect cells expressing hH_2R or gpH_2R and on isolated spontaneously beating guinea pig right atrium (Xie *et al.*, 2006; Ghorai *et al.*, 2008; Kraus *et al.*, 2009; Birnkammer, 2011). The intention of this study was firstly, to assess a potential involvement of the N-terminus in behavior of these ligands at the hH_2R and gpH_2R and secondly, to characterize them in a physiologically relevant test system, on human neutrophil granulocytes.

4.4.1 Ambivalent role of the H_2R N-terminus

The N-terminus of many peptidergic GPCRs is involved in binding of large peptide ligands, e.g. in case of complement factor 5a receptor (Chen *et al.*, 1998) and chemokine receptor CXCR4 (Rana and Baranski, 2010). On the contrary, the N-terminus of the aminergic GPCRs is normally considered just as an inert element of the receptor and there are only few reports about its functional role (Strasser *et al.*, 2008).

Several studies were already performed in order to explain the species-selective profile of acylguanidines at the hH_2R and $gphH_2R$. Amino acids at positions 17 in TM I (Cys at the hH_2R and Tyr at the gpH_2R) and 271 in TM VII (Ala at the H_2R and Asp at the gpH_2R) (Fig. 4.1), forming interhelical H-bond at the gpH_2R but not at the hH_2R, are just partially responsible for distinct potencies and efficacies of monovalent and bivalent acylguanidine-type ligands between species (Preuss *et al.*, 2007a; Birnkammer, 2011). The second extracellular loop (E2-loop), reported to be in close contact to ligands in the orthosteric binding pocket for some other GPCRs (Palczewski *et al.*, 2000; Peeters *et al.*, 2011), was studied also at the H_2R. However, mutual exchange of four non-conserved amino acids at positions 167, 169, 171 and 172 of the hH_2R and the gpH_2R (Fig. 4.1), respectively, resulted in similar potencies and efficacies of monovalent N^G-acylguanidines at the mutant and corresponding wild-type receptors (Preuss *et al.*, 2007b). The E2-loop was also analyzed as a putative accessory binding site of bivalent ligands. Hence, mutagenesis studies revealed only minor influence of the E2-loop onto the potency of some bivalent 2-amino-4-methylthiazolyl ligands, but no effect with respect to other types of bivalent compounds (Birnkammer, 2011).

Molecular modeling of N-terminus of GPCRs is even more challenging than modeling of already highly flexible extracellular loops. Hence, mutagenesis studies are a better alternative to study this flexible domain. By the generation of receptor chimera $h_{gpNT}H_2R\text{-}G_{s\alpha S}$, no shift in charge was introduced in the area of N-terminus, but few

alterations in polarity and flexibility of this domain (Fig. 4.1). Because of the close proximity to the N-terminus also Cys-17 in the TM I of the hH_2R was replaced by Tyr-17 of the gpH_2R. As already mentioned before amino acid at position 17 together with amino acid at position 271 is involved in species-selective profile of N^G-acylguanidines (Preuss *et al.*, 2007a; Birnkammer, 2011), but because of the lack of Asp at position 271 in the $h_{gpNT}H_2R\text{-}G_{s\alpha S}$, a crucial interhelical H-bond cannot be formed. The characterization of N^G-acylguanidines at the $h_{gpNT}H_2R\text{-}G_{s\alpha S}$ indicates ambivalent influence of the altered N-terminus. Therefore, no general trend for the behavior of examined ligands could be observed at the $h_{gpNT}H_2R\text{-}G_{s\alpha S}$ (Table 4.1).

The N-terminus of the hH_2R and gpH_2R on its own is not a key player in determining the species-selective profile of acylguanidines. Nevertheless, in a three-dimensional homology model of hH_2R a putative interaction of the second extracellular loop and N-terminus was suggested (Preuss *et al.*, 2007b). Moreover, activation of thyroid stimulating hormone receptor was influenced by cooperative action of more extracellular domains (Kleinau *et al.*, 2008). Molecular dynamic simulation at the hH_1R also revealed an importance of H-bond network between N-terminus, E1-loop and E2-loop for conformation and flexibility of the E2-loop (Strasser *et al.*, 2008). A complex network of interactions between extracellular domains should be kept in mind, when molecular determinants influencing the receptor activation process are discussed (Peeters *et al.*, 2011). The species-specificity, especially of the bivalent N^G-acylated hetarylpropylguanidines, cannot be explained by exchanging only the N-terminus of the H_2R. But it can be speculated that an interaction between the N-terminus and two or more extracellular loops is prerequisite for the high activity at the gpH_2R.

4.4.2 Unexpected effects of N^G-acylated hetarylpropylguanidines on neutrophil granulocytes

Although monovalent and bivalent N^G-acylated hetarylpropylguanidines are very potent H_2R agonists and therefore interesting tools for further pharmacological studies, these compounds have some drawbacks. Because of their cationic-amphiphilic character, they are susceptible to interaction with biological membranes and off-targets (Schreier *et al.*, 2000). Hemolytic activity was detected for several rather lipophilic monovalent acylguanidines at a concentration of 100 µM and for bivalent ligands with a longer lipophilic spacer ($n \geq 14$) at a concentration of 10 µM (Birnkammer, 2011). Cytotoxic effects were detectable at concentrations ≥ 10 µM (Birnkammer, 2011). Acylguanidines also bound strongly to serum albumin (generally ≥ 90 %) (Birnkammer, 2011). The aforementioned properties of the title compounds have to be taken into consideration, when studies on the cellular level or *in vivo* are planned.

In general, the development of bivalent ligands as potential drugs is compromised by unfavorable physicochemical properties (bulkyness and high molecular weight) and unfavorable pharmacokinetic properties (Shonberg *et al.*, 2011). However, besides their usefulness as pharmacological tools for investigation of receptor dimers *in vitro*, several examples from literature demonstrate that the bivalent ligand approach can result also in beneficial effects *in vivo*. One prominent example is a study published by Daniels *et al.* (2005). Hybrid compounds combining the pharmacophoric moieties of both the δ-opioid receptor antagonist naltrindole, reported to suppress antinociceptive tolerance, and the μ-opioid receptor agonist oxymorphone, connected with a 21-atom linker, revealed improved antinociceptive properties in mice compared to morphine. Additionally, this opioid receptor ligand did not induce tolerance and physical dependence upon chronic administration. Moreover, *in vitro* and *in vivo* studies on bivalent ligand with two sumatriptan molecules as pharmacophores revealed superior potency at 5-HT_{1B} receptor compared to the monovalent counterpart sumatriptan, which is currently used in the treatment of migraine (Perez *et al.*, 1998). These examples prove that the applicability of bivalent ligands is not restricted to recombinant test systems.

The effects of the acylguanidines on neutrophil granulocytes are difficult to explain. Neutrophil granulocytes are a much more complex test system for the characterization of H_2R agonists than Sf9 insect cell membranes, where only the receptor of interest is expressed. Previously, reduced potency in the $O_2^{\bullet-}$ assay on human neutrophil granulocytes (Burde *et al.*, 1990) compared to the GTPase assay on hH_2R-$G_{s\alpha S}$ (Preuss *et al.*, 2007b) was reported for the monovalent guanidines IMP and ARP. The potency of IMP and ARP differed 13- and 140-fold, respectively, between both assays. Nevertheless, IMP and ARP were still full agonists in the $O_2^{\bullet-}$ assay (Burde *et al.*, 1990) whereas bivalent N^G-acylguanidines were lacking of any agonistic activity at a concentration (1 μM) corresponding to – in some cases - more than 100-fold the EC_{50} value determined in the GTPase assay (Table 4.3). Moreover, inhibition of fMLP-stimulated $O_2^{\bullet-}$ production by the guanidine ARP could be competitively antagonized by famotidine (Burde *et al.*, 1990) whereas, in our study, the agonistic effect of acylguanidine **1** could not be reversed by the same H_2R antagonist (Fig. 4.5).

Monovalent as well as bivalent ligands stimulated cAMP accumulation in neutrophil granulocytes in a H_2R-dependent manner, although with reduced potency and efficacy compared to that observed in a recombinant test system (Table 4.2). Results of structure-activity relationship studies with bivalent acylguanidines at the hH_2R-$G_{s\alpha S}$ (Birnkammer, 2011) are - at least in part - in agreement with the results of the cAMP assay on neutrophil granulocytes. A decanedioyl spacer between pharmacophoric groups is the common structural motif of the most potent H_2R agonists in both test systems.

It should be noted here that due to the disappointing results regarding effects of bivalent N^G-acylguanidines in functional assays on neutrophil granulocytes, we were aiming at detecting of hH_2R molecules on these cells. In our laboratory, red fluorescent H_2R ligands were synthesized and successfully characterized on HEK293 cells, expressing recombinant hH_2Rs (Erdmann, 2011). Unfortunately, preliminary experiments revealed unsuitability of these ligands for binding studies on neutrophil granulocytes. Unexpectedly, flow cytometric experiments and confocal microscopy were hampered by penetration of the fluorescent H_2R ligands into the cells as well as by pronounced red autofluorescence of neutrophils (Lopuch, 2011). The presence of phagocyte-specific cytochrome b_{-245} (absorption maxima at 426, 528 and 558 nm) in neutrophils could at least partially account for the latter problem (Seifert and Schultz, 1991).

Another concern with respect to different activities of the title compounds in different test systems is the artificial nature of the hH_2R-$G_{s\alpha S}$ fusion protein, expressed in Sf9 cells, resulting in high potencies. Nevertheless, in the study of Xie and coworkers, IMP and ARP were examined at the hH_2R-$G_{s\alpha S}$ fusion protein in GTPase assay and at non-fused hH_2R in adenylate cyclases assay, using Sf9 cells as expression system in both cases (Xie *et al.*, 2006). The potencies of two above-mentioned ligands were even ~2.5-fold higher in adenylyl cyclase assay than in GTPase assay, ruling out the role of the $G_{s\alpha S}$ as a potency-intensifier at least in case of the guanidines. Moreover, representative bivalent acylguanidines were characterized also at the spontaneously beating guinea pig right atrium, which is a standard organ for the investigation of H_2R ligands. Here, the EC_{50} and E_{max} values of the most potent agonists correlated well with the data, determined at the gpH_2R-$G_{s\alpha S}$ in recombinant test system (Birnkammer, 2011).

As mentioned before, inhibition of fMLP-stimulated $O_2^{\bullet-}$ production in neutrophil granulocytes is a very indirect way of assessing H_2R agonistic activity. Upon exposure to fMLP, formyl peptide receptors are activated and couple to G_i proteins (Gierschik *et al.*, 1989; Wenzel-Seifert *et al.*, 1999). Further signaling events including activation of phospholipase C and protein kinase C result in $O_2^{\bullet-}$ production, catalyzed by NADPH oxidase (Burde *et al.*, 1989). Activation of the H_2R leads to cAMP increase and $O_2^{\bullet-}$ production is presumably inhibited by cAMP-dependent protein kinases (Seifert and Schultz, 1991). In this complex signaling event, where NADPH oxidase can be activated by numerous stimuli acting through different pathways, not only *via* formyl peptide receptors (Seifert and Schultz, 1991), it is difficult to find an explanation for unspecific (non-H_2R-mediated) suppression of $O_2^{\bullet-}$ production by amphiphilic monovalent acylguanidine **1** (Fig. 4.5). It is known from the literature that lipophilic ARP-like guanidines and cationic amphiphilic HA-derivative are capable of stimulating G_i proteins in a receptor-independent manner, resulting in $O_2^{\bullet-}$ elevation in neutrophils and HL-60 cells (Hagelüken *et al.*, 1995). Also in general, numerous

cationic amphiphilic molecules are known to stimulate G proteins in a receptor-independent way by passing cell membrane (Mousli *et al.*, 1990). Thus, compound **1** may be able to enter the cell and inhibit $O_2^{\bullet-}$ generation on different levels of the above described cascade of events. Furthermore, the direct interaction of basic heterocycle of compound **1** with cytochrome b_{-245} of neutrophils or ferricytochrome c cannot be excluded. The former will result in reduced $O_2^{\bullet-}$ production (Seifert and Schultz, 1991), the latter in diminished reduction of ferricytochrome c (Burde *et al.*, 1990). In both cases the results can be mis-interpretated as H_2R agonistic activity. Further studies on this topic would be needed to explain the effect of compound **1**.

Another point that should be discussed are tissue-specific properties of H_2Rs. Remarkable dissociation of H_2R-mediated agonistic effects was observed for the series of IMP analogues between guinea pig right atrium and guinea pig gastric fundus (Sterk *et al.*, 1987). Moreover, also within one organ, different behavior of H_2R ligands was reported. The positive inotropic response in guinea pig papillary muscle differed from positive chronotropic effect in the isolated guinea pig right atrium for some ARP-like H_2R agonists (Buschauer, 1989). Tissue specific behavior of one and the same receptor subtype can be influenced by different compartmentation of signaling partners at the plasma membrane (Lasley, 2011), by formation of homo- or heteromers (Smith and Milligan, 2010), by variable susceptibility to receptor desensitization and internalization (Tobin *et al.*, 2008) as well as by different expression level and receptor reserve. Additionally, surface properties of different cell types are also highly relevant in case of amphiphilic molecules. The selectivity of amphiphilic peptides for some cell types strongly depends on extent of the negative charge on the cell surface (Mousli *et al.*, 1990). For example, negatively charged sialic acid residues on the cell surface were suggested to be involved in interaction of amphiphilic substance P with mast cell membrane (Mousli *et al.*, 1989). Sialic acid content dynamically changes during maturation and activation process of neutrophil granulocytes (Cross *et al.*, 2003; Sakarya *et al.*, 2004). It is conceivable, that interactions of amphiphilic acylguanidines with sialic acid and other negatively charged residues on the surface of neutrophil granulocytes diminishes number of ligands, which can specifically interact with H_2R, resulting in reduced potency. Therefore, acylguanidines have less perspective as anti-inflammatory drugs, but might be useful in the treatment of acute congestive heart failure or have beneficial effect in other tissues expressing H_2R. The next rational step would be the characterization of N^G-acylated hetarylpropylguanidines at the guinea pig neutrophil granulocytes to get more insight into the tissue specific behavior of those ligands at the same species. However, the limiting factor with respect of extensive studies is isolation of sufficient amounts of neutrophils from this small laboratory animal.

4.4.3 Conclusions

The aim of this study was to broaden our knowledge about the pharmacological profile of potent H_2R agonists, monovalent and bivalent N^G-acylated hetarylpropylguanidines. Mutagenesis study revealed rather marginal contribution of the N-terminus to the species-selective profile of acylguanidines at the hH_2R and gpH_2R. Furthermore, the characterization of title compounds on neutrophil granulocytes revealed (I) unspecific inhibition of fMLP-stimulated $O_2^{\bullet-}$ production by monovalent ligand **1**, (II) lack of inhibitory effect of bivalent acylguanidines on fMLP-stimulated $O_2^{\bullet-}$ production and (III) weak H_2R-mediated stimulation of cAMP production by monovalent and bivalent ligands compared to the Sf9/baculovirus system. In conclusion, further *in vitro* investigations and *in vivo* studies of representative compounds are needed to assess the potential therapeutic value of acylguanidine-type H_2R agonists.

4.5 References

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Chapter 5

Role of the second and third extracellular loops of the histamine H_4 receptor in receptor activation

Adapted from:

Brunskole I, Strasser A, Seifert R and Buschauer A (2011) Role of the second and third extracellular loops of the histamine H_4 receptor in receptor activation.

Naunyn Schmiedeberg's Arch Pharmacol 384:301-317.

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5.1 Abstract

The histamine H_4 receptor subtype (H_4R) belongs to the class 1 of G protein-coupled receptors and is involved in inflammatory and immunological processes. The aim of this study was to elucidate the importance of extracellular regions for the large species differences between the human (h) and canine (c) H_4R. Therefore, chimeric receptors were generated by replacing corresponding domains of the hH_4R with the canine N-terminus ($h_{cNT}H_4R$) and three canine extracellular loops, respectively ($h_{cE1}H_4R$, $h_{cE2}H_4R$ and $h_{cE3}H_4R$). Wild-type and chimeric H_4 receptors were expressed in Sf9 insect cells and subsequently characterized in [^{3}H]histamine binding experiments and in steady-state GTPase activity assays, where standard H_4R ligands histamine, 5-methylhistamine, thioperamide, 1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine (JNJ7777120) and clozapine were examined. The exchange of N-terminus or first extracellular loop did not influence hH_4R pharmacology. The effect of altered second extracellular loop (E2-loop) and third extracellular loop (E3-loop) was rather ligand-specific than agonist/inverse agonist-specific. At the $h_{cE3}H_4R$, the potency of histamine and 5-methylhistamine significantly decreased. The efficacy of the inverse agonist thioperamide was strongly reduced at the $h_{cE2}H_4R$ and the $h_{cE3}H_4R$. Surprisingly, JNJ7777120 as weak inverse agonist at the hH_4R exhibited partial agonistic activity at the $h_{cE2}H_4R$ and the $h_{cE3}H_4R$. Molecular dynamic simulations suggest that the E2- and E3-loops are independently of each other involved in partial/inverse agonism of JNJ7777120 and that E2- as well as E3-loop do not directly interact with JNJ7777120 in the binding pocket. In conclusion, our study indicates an involvement of E2- and E3-loop in H_4R activation process after binding of some but not all examined ligands.

5.2 Introduction

There are four histamine receptor subtypes known (histamine H_1, H_2, H_3 and H_4 receptor), which belong to the class 1 (rhodopsin-like class) of G protein-coupled receptors (GPCR) (Foord *et al.*, 2005; Thurmond *et al.*, 2008). The latest identified histamine receptor subtype, histamine H_4 receptor (H_4R), couples to pertussis toxin-sensitive $G\alpha_{i/o}$ proteins, leading to increases in intracellular calcium and inhibition of cAMP production (Thurmond *et al.*, 2008; Leurs *et al.*, 2009). The H_4R is mainly expressed on cells of haematopoietic origin, like mast cells, dendritic cells, monocytes, eosinophils, basophils and T cells, and thereby plays a role in modulation of immune and inflammatory processes (Thurmond *et al.*, 2008; Zampeli and Tiligada, 2009). The H_4R is regarded as a very promising drug target in respect of therapy of asthma, chronic pruritus and autoimmune disorders like rheumatoid arthritis (Zampeli and Tiligada, 2009).

Full-length human H_4R (hH_4R) consists of 390 amino acids, with an extracellular N-terminus, seven transmembrane domains (TM I-VII), intertwined by three extracellular and three intracellular loops, and an intracellular C-terminus. One characteristic of the hH_4R is quite low amino acid homology to other species, for instance 65 %, 68 % and 69 % to the guinea pig, mouse and rat H_4R, respectively (Liu *et al.*, 2001). These structural differences are translated into differing pharmacological profiles (Lim *et al.*, 2010; Schnell *et al.*, 2011). Consequently, the evaluation of H_4R ligands, potentially beneficial in therapy of humans, is limited in animal models. Thus, there is a need to identify structural determinants, responsible for differences in pharmacology of the hH_4R and H_4R of other species, used as animal models.

In 2008, the canine H_4R (cH_4R) from *Canis familiaris* was cloned (Jiang *et al.*, 2008). Testing of standard H_4R ligands at the cH_4R revealed major differences in binding affinity and potency compared to the hH_4R (Jiang *et al.*, 2008; Lim *et al.*, 2010; Schnell *et al.*, 2011). Very recently, also an agonistic activity of thioperamide (THIO) and 1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine (JNJ7777120) at the cH_4R, expressed in Sf9 insect cells, was reported (Schnell *et al.*, 2011). These two ligands exhibit inverse agonistic activity at the hH_4R. The cH_4R is composed of 395 amino acids and shares 71 % of an overall sequence identity with the hH_4R and 83 % of sequence identity in transmembrane area (Fig. 5.1A). Many amino acids in transmembrane regions of the hH_4R, identified as crucial for histamine (HA) binding, receptor activation and G-protein coupling, are identical at the cH_4R (Jiang *et al.*, 2008). This gives rise to a question, whether the regions other than transmembrane domains are important for species differences, observed for the hH_4R and the cH_4R.

For numerous aminergic GPCRs, like α_{1A}- and α_{1B}-adrenergic receptors, the β_2-adrenergic receptor, M_1, M_2, M_3 and M_4 muscarinic acetylcholine receptors, 5-HT_{1D} and 5-HT_{1B} receptors, adenosine A_{2A} and A_{2B} receptors as same as the histamine H_1 receptor (Shi and Javitch, 2002; Lawson and Wheatley, 2004; Strasser *et al.*, 2008b; Peeters *et al.*, 2011a; Peeters *et al.*, 2011b), extracellular regions are functionally important parts of the receptor. Most importantly, small-molecule ligands with different modes of action (agonists, antagonists or inverse agonists) stabilize distinct extracellular surface conformations of β_2-adrenergic receptor, indicative of a role of extracellular domains as modulators of receptor function (Bokoch *et al.*, 2010). In case of the H_4R, comparison of four extracellular regions of human and canine receptor revealed sequence identity of 63 %, 55 %, 48 % and 20 % for N-terminus, first extracellular loop (E1-loop), second extracellular loop (E2-loop) and third extracellular loop (E3-loop), respectively. It is also noteworthy, that the E2-loop of the cH_4R is six amino acids longer than the E2-loop of the hH_4R (Fig. 5.1A). Therefore, in the present

study, we were aiming at evaluating the relevance of differing extracellular domains for differences in the pharmacology of the hH_4R and the cH_4R.

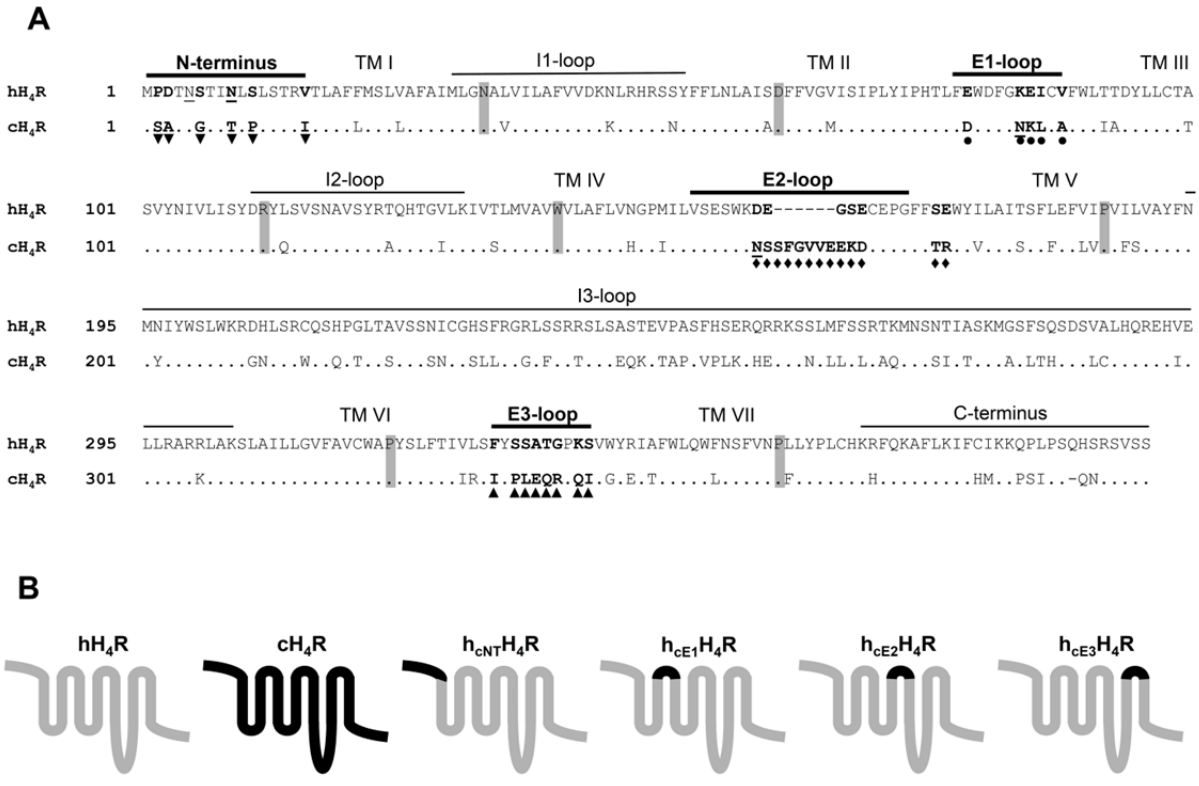

Fig. 5.1 Human, canine and chimeric histamine H_4 receptors. (**A**) Alignment of the amino acid sequences of the wild-type hH_4R and cH_4R (Jiang *et al.*, 2008). TM I-VII, seven transmembrane domains; I1-3 loop, first, second and third intracellular loop; E1-3 loop, first, second and third extracellular loop. Dots in the sequence of the cH_4R indicate identical amino acids with the hH_4R. Hyphens stand for missing amino acids and underlined amino acids indicate putative glycosylation sites in extracellular regions. Amino acids with grey shading represent the most conserved amino acids among aminergic G protein-coupled receptors (Shi and Javitch, 2002). Amino acids in bold were exchanged by the generation of chimeric H_4 receptors; $h_{cNT}H_4R$, with downward-facing triangle (▼) marked amino acids at the hH_4R were replaced by corresponding amino acids of the cH_4R; $h_{cE1}H_4R$, with circle (●) marked amino acids at the hH_4R were replaced by corresponding amino acids of the cH_4R; $h_{cE2}H_4R$, with diamond (♦) marked amino acids at the hH_4R were replaced by corresponding amino acids of the cH_4R or inserted; $h_{cE3}H_4R$, with upward-facing triangle (▲) marked amino acids at the hH_4R were replaced by corresponding amino acids of the cH_4R. (**B**) Schematic depiction of wild-type hH_4R, cH_4R as well as generated H_4R chimeras. The parts of the human H_4R are shown in grey and the parts of the canine H_4R are shown in black.

For this reason, we generated chimeric H_4R receptors by replacing corresponding extracellular domains of the hH_4R with the canine N-terminus ($h_{cNT}H_4R$) and three canine extracellular loops, respectively ($h_{cE1}H_4R$, $h_{cE2}H_4R$ and $h_{cE3}H_4R$) (Fig. 5.1B). Wild-type hH_4R and cH_4R as well as chimeric H_4 receptors were expressed in Sf9 insect cells together with G protein subunits $G\alpha_{i2}$ and $G\beta_1\gamma_2$ and characterized in [^{3}H]HA binding experiments and in steady-state GTPase activity assays. Here, standard H_4R ligands HA, 5-methylhistamine (5-

MHA), THIO, JNJ7777120 and clozapine were tested (Fig. 5.2). To obtain detailed insight into the influence of the E2-loop and E3-loop with regard to ligand binding and receptor activation, molecular modelling studies were performed.

5.3 Materials and Methods

5.3.1 Materials

The pcDNA 3.1 plasmid with the sequence of the hH_4R was obtained from UMR cDNA Resource Center at the University of Missouri-Rolla (Rolla, MO). The pCIneo plasmid containing the sequence of the cH_4R was kindly provided by Dr. R. Thurmond (Johnson & Johnson Pharmaceutical R&D, San Diego, CA). The generation of pGEM-3Z-SF-hH_4R-His_6, pVL1392-SF-hH_4R-His_6-$G\alpha_{i2}$ and pVL1392-SF-cH_4R-His_6 was described previously (Schneider *et al.*, 2009; Schnell *et al.*, 2011). Baculovirus encoding $G\alpha_{i2}$ was donated by Dr. A. G. Gilman (Department of Pharmacology, University of Southwestern Medical Center, Dallas, TX). Baculovirus encoding the unmodified versions of the $G\beta_1\gamma_2$ subunits was kindly provided by Dr. P. Gierschik (Department of Pharmacology and Toxicology, University of Ulm, Germany). The anti-Flag IgG (M1 monoclonal antibody) was from Sigma (St. Louis, MO). The antibody selective for $G\alpha_{i1/2}$ was purchased from Calbiochem (La Jolla, CA). The DNA primers for PCR were purchased by MWG Biotech (Ebersberg, Germany). Phusion high-fidelity DNA Polymerase was obtained from Finnzymes (Espoo, Finland). Restriction enzymes and T4-DNA ligase were from New England Biolabs (Ipswich, MA). [^{3}H]HA (14.2 Ci/mmol) was from Perkin Elmer (Boston, MA). [γ-^{32}P]GTP was synthesized using GDP and [γ-^{32}P]P_i (8,500-9,100 Ci/mmol orthophosphoric acid) as described previously (Walseth and Johnson, 1979). Analogously, [γ-^{33}P]GTP was synthesized using GDP and [γ-^{33}P]P_i (3,000 Ci/mmol orthophosphoric acid). GDP was purchased from Sigma-Aldrich (St. Louis, MO), [γ-^{32}P]P_i from Perkin Elmer (Boston, MA) and [γ-^{33}P]P_i from Hartmann Analytic (Braunschweig, Germany). All other reagents were from standard suppliers and had the highest purity available. Radioactive samples were counted in a PerkinElmer Tricarb 2800TR liquid scintillation analyzer.

HA, 5-MHA and THIO were obtained from Tocris (Avonmouth, Bristol, UK), clozapine from Research Biochemicals International (Natick, MA) and JNJ7777120 from Axon Medchem (Groningen, Netherlands). Chemical structures of ligands are depicted in Fig. 5.2. Stock solutions and dilutions of HA, 5-MHA and THIO were prepared in millipore water. 10 mM stock solutions of JNJ7777120 and clozapine were dissolved in dimethyl sulfoxide (DMSO) and dilution series, used in assays, were prepared in 10 % DMSO (v/v) to achieve final DMSO concentration of 1 % (v/v) in each assay tube.

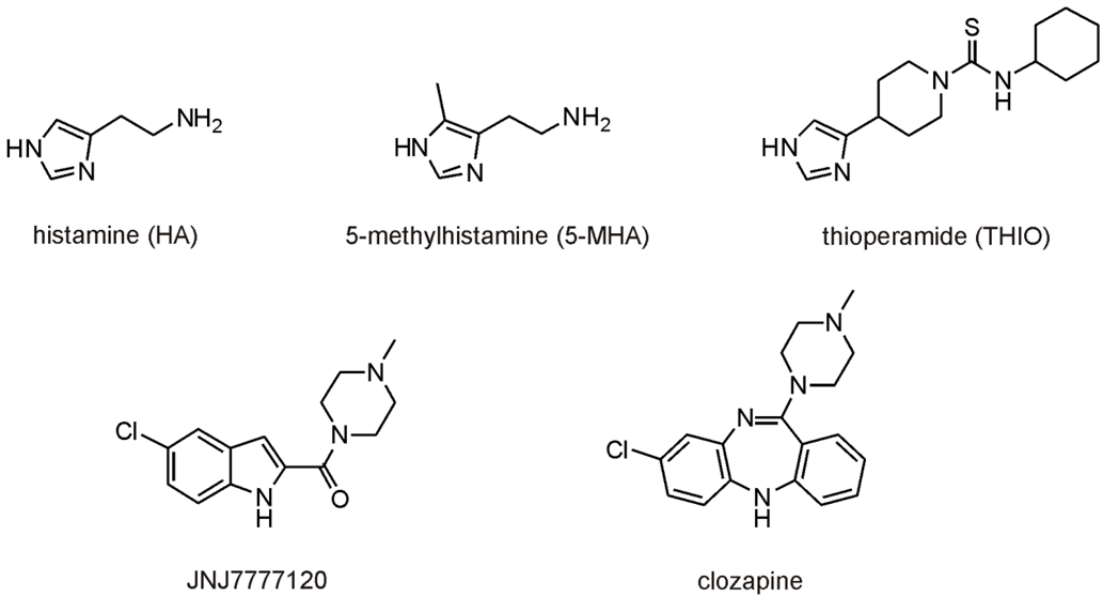

Fig. 5.2 Structures of the tested H_4R ligands. Using Sf9 insect cells as expression system and steady-state GTPase activity assay as read-out, histamine (HA), 5-methylhistamine (5-MHA) and clozapine show agonistic activity at the hH_4R as same as at the cH_4R. In contrast, thioperamide (THIO) and JNJ7777120 exhibit species-specific mode of action; they act as inverse agonists at the hH_4R and as agonists at the cH_4R (Schnell *et al.*, 2011).

5.3.2 Construction of the cDNA for the $h_{cNT}H_4R$

The cDNA for the $h_{cNT}H_4R$ was generated by sequential overlap-extension PCR, using pGEM-3Z-SF-hH_4R-His_6 as template, by analogy with approaches described before (Preuss *et al.*, 2007; Strasser *et al.*, 2008b). In PCR 1A, the DNA region encoding for cleavable signal peptide from influenza hemagglutinin (S; ATGAAGACGATCATCGCCCT GAGCTACATCTTCTGCCTGGTATTCGCC), the FLAG epitope (F; GACTACAAGGACGAT GATGACGCC) recognized by the M1 monoclonal antibody and the N-terminus of cH_4R was amplified. The sense primer annealed with 24 bp of pGEM-3Z prior to the 5'-end of SF. The antisense primer with the sequence 5'-***T***ACG***C***GTGCTTAGTG***G***TAAA***G***TGATTGT ***T***C***C***ATTAGTA***G***CTG***A***CATGGCGTCATCATCGTCCTTGTAG-3' was used to generate Pro2→Ser2, Asp3→Ala3, Ser6→Gly6, Asn9→Thr9, Ser11→Pro11 and Val16→Ile16 mutations in the region of N-terminus and a new *Mlu* I restriction site (ACGCGT). In PCR 1B, the DNA sequence of canine N-terminus, the rest of the hH_4R and a hexahistidine tag (CACCATCATCACCATCAC) was amplified. The hexahistidine tag allows future purification and provides additional protection against proteolysis. The sense primer encoded the sequence 5'-***T***CAG***C***TACTAAT***G***G***A***ACAATCA***C***TTTA***C***CACTAAGCAC***G***CGT***A***TTACTTT AGCATTTTTTATGTCCTTAGTAG-3' to generate Pro2→Ser2, Asp3→Ala3, Ser6→Gly6, Asn9→Thr9, Ser11→Pro11 and Val16→Ile16 mutations and a new *Mlu* I restriction site. The antisense primer annealed with 18 bp of pGEM-3Z after the stop codon and after an *Xba* I

site. In PCR 2, using the sense primer of PCR 1A and the antisense primer of PCR 1B, the products of PCR 1A and 1B annealed in the region of newly created canine N-terminus and a new *Mlu* I restriction site. The product of PCR 2 encoded the complete cDNA for SF-$h_{cNT}H_4R$-His_6 protein. This product was double-digested with *Sac* I and *Xba* I and cloned into pGEM-3Z-SF-hH_4R-His_6 digested with the same enzymes. pGEM-3Z-SF-$h_{cNT}H_4R$-His_6 was digested with *Sac* I and *Xba* I and cloned into the baculovirus transfer vector pVL1392-SF-hH_4R-His_6 digested with the same enzymes. The sequence of SF-$h_{cNT}H_4R$-His_6, cloned into the pGEM-3Z plasmid, was verified by restriction enzyme analysis and sequencing (Entelechon, Regensburg, Germany).

5.3.3 Construction of the cDNA for the $h_{cE1}H_4R$, $h_{cE2}H_4R$, $h_{cE3}H_4R$ and $h_{cE3}H_4R$-$G\alpha_{i2}$.

The cDNAs for the $h_{cE1}H_4R$, $h_{cE2}H_4R$ and $h_{cE3}H_4R$ were generated by analogy with the generation of cDNA for the $h_{cNT}H_4R$, using pGEM-3Z-SF-hH_4R-His_6 as template. For construction of the $h_{cE1}H_4R$ cDNA, the antisense primer 5'-***G***C***G***CAGA***G***TT***TA***TTTCCAAAA TCCCA***A***TCGAA CAGCGTGTGAGGGATG-3' was used in PCR 1A and the sense primer 5'-***T***TGGGATTTTGGAAA***TA***AA***C***TCTG***C***G***C***ATTTTGGCTCACTACTGACTATCTG-3' was used in PCR 1B in order to generate mutations Glu79→Asp79, Lys84→Asn84, Glu85→Lys85, Ile86→Leu86 and Val88→Ala88 in the region of E1-loop and a new *Fsp* I restriction site (TGCGCA).

In case of the $h_{cE2}H_4R$, the antisense primer 5'-***CCTG***G***TG***AAAAATCCAGGTTC ACA***A***TC***CT***T***CT***C***CTCCACGACTCCGAAACTGGA***AT***T***CTTCCAAGACTCTGAAACTAGAA TCATTGG-3' was used in PCR 1A and the sense primer 5'-***A***AT***TCCAGTTTCGGAGTC GTGGAG***G***A***G***A***AG***GA***T***TGTGAACCTGGATTTTT***CA***C***CAGG***TGGTACATCCTTGCCATCA CATCATTC-3' was used in PCR 1B in order to generate mutations Asp159→Asn159, Glu160→Ser160, Gly161→Glu167, Ser162→Lys168, Glu163→Asp169, Ser170→Thr176 and Glu171→Arg177 in the region of E2-loop and a new *Dra* III restriction site (CACNNNGTG) and to insert 6 amino acids in the region of E2-loop (Ser161, Phe162, Gly163, Val164, Val165, Glu166). Amino acids at positions 170 (Ser) and 171 (Glu) in the hH_4R are according to (Jiang *et al.*, 2008) located already in TM V (Fig. 5.1A), but were included in the mutagenesis study because of the close proximity to E2-loop.

For construction of the $h_{cE3}H_4R$ cDNA, the antisense primer 5'-***AT***TT***GT***GGAC ***GCTG***T***T***CT***AGT***G***G***ATAAA***T***TGAAAGGACAATTGTGAACAGAG-3' was used in PCR 1A and the sense primer 5'-***A***TTTAT***C***C***ACT***AG***A***A***CAGC***GTCC***AC***AA***AT***AGTTTGGTATAG AATTGCATTTTGG-3' was used in PCR 1B in order to generate mutations Phe328→Ile334, Ser330→Pro336, Ser331→Leu337, Ala332→Glu338, Thr333→Gln339, Gly334→Arg340, Lys336→Gln342 and Ser337→Ile343 in the region of E3-loop and a new *Xcm* I restriction site (CCANNNNNNNNTGG).

The same primers as by the generation of the $h_{cE3}H_4R$ were used in synthesis of cDNA for the fusion protein $h_{cE3}H_4R$-Gα_{i2}. However, here pVL1392-SF-hH_4R-His_6-Gα_{i2} was used as template.

5.3.4 Generation of recombinant baculoviruses, Sf9 insect cell culture and membrane preparation

Generation of recombinant baculoviruses and Sf9 insect cell culture was described recently (Schneider *et al.*, 2009). Before infection, Sf9 cells were sedimented and suspended in fresh medium. Cells were seeded at a density of 3.0 x 10^6 cells/ml and infected with high-titer baculovirus stocks encoding different histamine H_4 receptors, Gα_{i2} and G$\beta_1\gamma_2$ (1:100 dilution), if not indicated otherwise. Cells were cultured for 48 h at 28 °C under rotation at 150 rpm and afterwards, membrane preparation was performed as described (Seifert *et al.*, 1998a). Here, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 µg/ml benzamidine, and 10 µg/ml leupeptin were used as protease inhibitors. Membranes were suspended in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4) and stored in aliquots at -80 °C.

In order to analyze the effect of HA or THIO on the expression level of the hH_4R and the $h_{cE2}H_4R$, mini-membrane preparations were conducted as reported recently (Schneider *et al.*, 2009). Briefly, 15 x 10^6 Sf9 cells were seeded in 15 ml cell culture medium (75 cm^2 culture flasks). By control expression, no ligand was added to the flask. To other flasks, 100 µM THIO or 1 mM HA solutions were added to yield final concentration of 1 µM for THIO or 10 µM for HA, respectively. Furthermore, the baculovirus stocks (hH_4R or $h_{cE2}H_4R$ + Gα_{i2} + G$\beta_1\gamma_2$) were added to achieve 1:100 dilution. Cells were cultured for 48 h at 28 °C. HA (10 µM) was additionally added after 24 h to decompensate degradation. After 48 hours, cells were harvested, centrifuged and washed with phosphate-buffered saline. After additional centrifugation, the cells were homogenised in 0.5 ml lysis buffer, used also by regular membrane preparation. Then, centrifugation and additional wash step with 1 ml lysis buffer followed. At the end, membranes were re-suspended in 2 ml binding buffer and stored in aliquots at -80 °C. Three washes during mini-membrane preparation and one additional wash step before performance of [^{3}H]HA binding experiments and steady-state GTPase activity assays assured removal of HA or THIO from the membranes.

5.3.5 [^{3}H]HA binding experiments

Prior to experiments, Sf9 membranes were thawed and sedimented by a 10 min centrifugation step at 4 °C and 13,000 rpm. Membranes were then re-suspended in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4). Each tube contained 100

µg of protein in a total volume of 250 µl or 200 µg protein in a total volume of 500 µl. For saturation binding, Sf9 membranes were incubated in binding buffer in the presence of [^{3}H]HA (0.78-100 nM) and 0.2 % (m/v) bovine serum albumin. For competition binding, Sf9 membranes in binding buffer, 20 nM [^{3}H]HA, 0.2 % (m/v) bovine serum albumin and examined ligands at various concentrations were used. Non-specific binding was determined in the presence of 100 µM THIO. Incubations were performed for 60 min at room temperature and 250 rpm. Bound [^{3}H]HA was separated from free [^{3}H]HA by filtration through GF/C filters, pretreated with 0.3 % (m/v) polyethyleneimine, followed by three washes with 2 ml ice-cold binding buffer (4 °C). Filter-bound radioactivity was measured by liquid scintillation counting.

5.3.6 Steady-state GTPase activity assay

Steady-state GTPase activity assays, using [γ-^{32}P]GTP as radioligand (0.1 µCi per tube), were essentially conducted as already described (Preuss *et al.*, 2007), but with 5.0 mM $MgCl_2$, 1.2 mM creatine phosphate and 1 µg creatine kinase in the samples. Each sample contained 10-20 µg of membrane protein. GTPase activity was measured in the presence of 100 mM NaCl. In the same manner, some experiments were performed using [γ-^{33}P]GTP as radioligand (0.05 µCi per tube). No shift in pEC_{50} and E_{max} values was observed after substitution of [γ-^{32}P]GTP with [γ-^{33}P]GTP.

5.3.7 Molecular Modelling

For construction of several receptor models, the technique of homology modelling was used. Based on the crystal structure of the human β_2-adrenergic receptor (protein data bank code 2RH1) (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Rosenbaum *et al.*, 2007) a homology model of the inactive hH_4R was generated as described previously (Deml *et al.*, 2009; Schneider *et al.*, 2010). For construction of the homology models of the active cH_4R, $h_{cE2}H_4R$ and $h_{cE3}H_4R$ the crystal structure of opsin co-crystallized with the C-terminal part of Gα (protein data bank code 3DQB) (Scheerer *et al.*, 2008) was used as a template. The most recent crystal structure of an active state of the β_2-adrenergic receptor, 3P0G (Rasmussen *et al.*, 2011), is nanobody-stabilized. This nanobody, interacting with the receptor in the intracellular part, is more voluminous than the C-terminus of Gα, which interacts with a GPCR. Thus, the outward movement of TM VI is larger in 3P0G than in 3DQB. This may lead to a structural artefact in the intracellular part of the receptor. Furthermore, unnatural intracellular part may have influence onto the conformation of the whole receptor. Thus, we decided to use opsin as template for modelling the active state conformation of H_4Rs.

JNJ7777120 was docked manually into the binding pocket of each receptor model. Subsequently, molecular dynamic (MD) simulations were performed, as described previously (Strasser *et al.*, 2008a), including the natural surroundings of the receptor, like lipid bilayer and water. Simulation parameters were used as described (Strasser *et al.*, 2008a). For all MD simulations, the software Gromacs (http://www.gromacs.org) was used (Oostenbrink *et al.*, 2004).

5.3.8 Miscellaneous

Protein concentrations were determined using Bio-Rad DC protein assay kit (Hercules, CA). SDS-PAGE and immunoblot analysis was performed as described (Schneider *et al.*, 2009). Data obtained from radioligand binding and GTPase experiments were analysed with the Prism 5.01 software (GraphPad, San Diego, CA). All values are given as mean ± S.E.M. of at least three independent experiments. If not indicated otherwise, significance was calculated using one-way ANOVA, followed by Dunnett's or Bonferroni's multiple comparison test. Data from Fig. 5.4 were analyzed with Dunnett's method in order to compare B_{max} value for the hH_4R or the $h_{cE2}H_4R$ after expression in the absence of ligands (reference value) with B_{max} value after expression in the presence of HA or THIO for the same receptor. By analogy, data from Table 5.1 were also analyzed with Dunnett's method in order to compare relative GTPase activities of the hH_4R or the $h_{cE2}H_4R$, respectively, after expression under different conditions. Data from Table 5.2 (K_D and B_{max} values), Table 5.3 (pK_i values) and Table 5.4 (pEC_{50} and E_{max} values) were analyzed with Bonferroni's method, where constants (K_D, B_{max}, pK_i, pEC_{50} and E_{max}, respectively) at different receptors (hH_4R, cH_4R and four H_4R chimeras) were compared with each other. Statistical significance was defined as $p < 0.05$ (95 % confidence interval).

5.4 Results

5.4.1 Immunological detection of recombinant proteins

Histamine H_4 wild-type receptors (hH_4R, cH_4R) and chimeric receptors ($h_{cNT}H_4R$, $h_{cE1}H_4R$, $h_{cE2}H_4R$, $h_{cE3}H_4R$) were expressed in Sf9 insect cells together with G protein subunits $G\alpha_{i2}$ and $G\beta_1\gamma_2$. All studied H_4 receptors were N-terminally tagged with FLAG-epitope and therefore could be detected with M1 anti-FLAG antibody. The hH_4R appeared as a diffuse band at ~40 kDa (Fig. 5.3A, lane 1), which is in agreement with literature data (Schneider *et al.*, 2009; Schneider and Seifert, 2009). Receptor chimeras were also well expressed and exhibited migration properties similar to those of the hH_4R (Fig. 5.3A, lane 3-

6). Nevertheless, the cH_4R migrated as two bands in SDS-PAGE and occurred at somewhat higher molecular masses than the diffuse band representing the hH_4R (Fig. 5.3A, lane 2).

The expression of $G\alpha_{i2}$ in membranes from the batch of Sf9 cells, used also for detection of FLAG-tagged H_4Rs (Fig 5.3A), was confirmed using anti-$G\alpha_{i1/2}$ antibody. $G\alpha_{i2}$ appeared at the expected molecular mass of ~41 kDa (Fig. 5.3B) (Kleemann *et al.*, 2008).

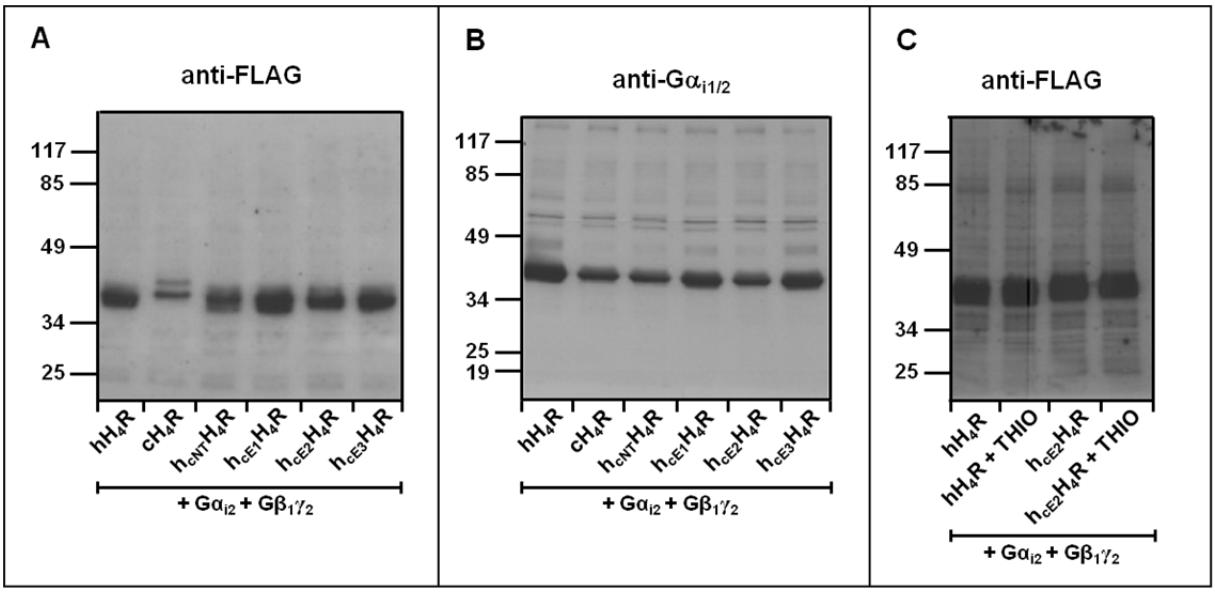

Fig. 5.3 Immunological analysis of recombinant proteins, expressed in Sf9 insect cell membranes. Sf9 cell membranes, expressing different histamine H_4 receptors together with $G\alpha_{i2}$ and $G\beta_1\gamma_2$, were analysed as described under 5.3.8. In each lane of the gels, 10 µg of protein was loaded. Membranes, shown in the same panel, are from the same membrane preparation. Numbers on the left of each panel indicate the molecular masses of marker proteins in kDa. (**A**) Detection of FLAG-tagged proteins hH_4R, cH_4R, $h_{cNT}H_4R$, $h_{cE1}H_4R$, $h_{cE2}H_4R$ and $h_{cE3}H_4R$, using M1 monoclonal antibody (anti-FLAG Ig). (**B**) Detection of $G\alpha_{i2,}$ using anti-$G\alpha_{i1/2}$ antibody. (**C**) Detection of hH_4R and $h_{cE2}H_4R$, expressed in Sf9 insect cells under control conditions and in the presence of 1 µM THIO, with M1 monoclonal antibody (anti-FLAG Ig).

5.4.2 Structural instability of the $h_{cE2}H_4R$

Expression of the $h_{cE2}H_4R$ was confirmed by immunoblotting, where the band representing the $h_{cE2}H_4R$ had only slightly reduced intensity compared to the band for the hH_4R (Fig. 5.3A, lane 1 and 5). However, preliminary [^{3}H]HA saturation binding experiments, using two membranes from independent membrane preparations, expressing the $h_{cE2}H_4R$ (+ $G\alpha_{i2}$ + $G\beta_1\gamma_2$), revealed very low specific binding of [^{3}H]HA. B_{max} value for the $h_{cE2}H_4R$ was 0.13 ± 0.01 pmol/mg, which is almost 15-fold lower than B_{max} for the hH_4R (1.77 ± 0.05 pmol/mg). Moreover, in steady-state GTPase assays, an agonistic effect of HA and inverse agonistic effect of THIO could be detected, but because of the low signal-to-noise ratio it was impossible to obtain reproducible pEC_{50} values. Those findings indicate a high structural instability of the chimeric $h_{cE2}H_4R$.

Some structurally labile receptors could be stabilized by expression in the presence of ligands (Gether *et al.*, 1997; Pauwels and Tardif, 2002; Roth *et al.*, 2008). Also, the agonist HA and the inverse agonist THIO were able to increase the B_{max} value of [^{3}H]HA binding to the wild-type hH_4R (Schneider *et al.*, 2009). Therefore, we decided to investigate the effect of ligands HA and THIO on stabilization of the structurally labile $h_{cE2}H_4R$ during expression. The hH_4R and the $h_{cE2}H_4R$ (+ $G\alpha_{i2}$ + $G\beta_1\gamma_2$) were expressed in Sf9 cells under control conditions, in the presence of 10 µM HA or in the presence of 1 µM THIO. Mini-membrane preparations were performed as described in section 5.3.4. Specific binding of [^{3}H]HA (100 nM) increased only slightly (~15 %), when the hH_4R was expressed with HA or THIO (Fig. 5.4). At the $h_{cE2}H_4R$, the amount of specific [^{3}H]HA binding sites was significantly (3.6 fold) higher after the expression in the presence of ligands (Fig. 5.4). While non-specific binding of [^{3}H]HA at the hH_4R was constantly 15 % of total binding, irrespective of expression conditions, the non-specific binding at the $h_{cE2}H_4R$ was reduced from 70 % (control expression) to 40 % (HA or THIO expression).

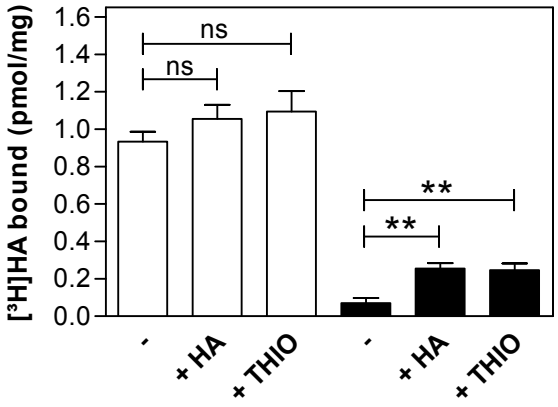

Fig. 5.4 Influence of different expression conditions on the B_{max} value of [^{3}H]HA binding to the hH_4R and the $h_{cE2}H_4R$. The hH_4R (white bars) and the $h_{cE2}H_4R$ (black bars) were expressed in Sf9 insect cells together with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in the absence of ligands (-), in the presence of 10 µM HA (+ HA) or in the presence of 1 µM THIO (+ THIO). Sf9 cell membranes (60 to 100 µg protein per tube) were incubated with 100 nM [^{3}H]HA as described in section 5.3.5. Data shown are the means ± S.E.M. of at least three independent experiments performed in triplicate. Membranes used were from independent mini-membrane preparations. B_{max} values were compared using one-way ANOVA, followed by Dunnett`s multiple comparison test; B_{max} value after expression in the presence of HA or THIO was compared with B_{max} value after expression in the absence of ligands (ns, not significant; **, $p < 0.01$).

Additionally, the effect of different expression conditions on maximal receptor stimulation (HA, 100 µM) and maximal receptor inhibition (THIO, 100 µM) was studied. As shown in Table 5.1, addition of ligands during the expression did not significantly improve relative HA-induced stimulation and THIO-induced inhibition of GTPase activity at the wild-

type hH_4R. At the $h_{cE2}H_4R$, small relative stimulatory effect of HA (+14.2 %, compared to basal GTPase activity) and small relative inhibitory effect of THIO (-5.12 %) in control expression was non-significantly increased by the expression in the presence of HA (+25.5 % and -11.7 %, respectively). Better improvement of signal-to-noise ratio was achieved by expression of the $h_{cE2}H_4R$ in the presence of THIO. Here, 100 µM THIO caused significantly stronger reduction of GTPase activity than at the $h_{cE2}H_4R$, expressed in the absence of THIO (-19.2 % *vs.* -5.12 %; $p < 0.05$). Furthermore, significant increase of GTPase activity after stimulation with 100 µM HA was observed, compared to the $h_{cE2}H_4R$, expressed under control conditions (+43.5 % *vs.* +14.2 %; $p < 0.001$).

Table 5.1 Influence of different expression conditions on the GTPase activity in Sf9 cell membranes expressing the hH_4R or the $h_{cE2}H_4R$.

GTPase activity	$hH_4R + G\alpha_{i2} + G\beta_1\gamma_2$		$h_{cE2}H_4R + G\alpha_{i2} + G\beta_1\gamma_2$			
	-	+ HA	-	+ HA	-	+ HA
basal	1.45	1.58	1.41	0.96	1.47	1.23
[pmol/(mg×min)]	± 0.25	± 0.10	± 0.17	± 0.17	± 0.12	± 0.26
ago. stim.	+ 21.4	+ 15.4	+ 23.3	+ 14.2	+ 25.5	+ 43.5
[% of basal]	± 2.46	± 2.69^{ns}	± 2.17^{ns}	± 2.48	± 0.55^{ns}	± 4.90***
inv. ago. inh.	- 20.8	- 20.0	- 26.6	- 5.12	- 11.7	- 19.2
[% of basal]	± 1.92	± 2.04^{ns}	± 3.79^{ns}	± 1.11	± 3.31^{ns}	± 5.73*

The hH_4R and the $h_{cE2}H_4R$ were expressed in Sf9 insect cells together with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in the absence of ligands (-), in the presence of 10 µM HA (+ HA) or in the presence of 1 µM THIO (+ THIO). Steady-state GTPase experiments were conducted as described in section 5.3.6. GTPase activity in Sf9 cell membranes was measured under basal conditions (basal), after maximal stimulation with an agonist (HA, 100 µM, ago. stim.) and after maximal inhibition with an inverse agonist (THIO, 100 µM, inv. ago. inh.). Membranes used were from independent mini-membrane preparations. Data shown are the means ± S.E.M. of three to four independent experiments performed in triplicate. Relative GTPase activity [% of basal] of the hH_4R and the $h_{cE2}H_4R$, respectively, after stimulation with an agonist or after inhibition with an inverse agonist was compared using one-way ANOVA, followed by Dunnett`s multiple comparison test; relative GTPase activity after expression in the presence of HA or THIO was compared with relative GTPase activity after expression in the absence of ligands (ns, not significant; *, $p < 0.05$; ***, $p < 0.001$).

Because THIO was superior to HA as an additive during receptor expression with respect to increasing the signal-to-noise ratio in GTPase assay, the $h_{cE2}H_4R$ expressed in the presence of THIO was further characterized. Immunoblot analysis revealed comparable expression levels of both the hH_4R and the $h_{cE2}H_4R$, recognized with M1 anti-FLAG antibody, independent of the presence or absence of THIO during expression (Fig. 5.3C). It is noteworthy, that not only properly folded receptors but also unfunctional receptors with accessible epitope are detected by the M1 anti-FLAG antibody. Unaltered expression level, assessed by immunoblotting, and increased B_{max} value of [^{3}H]HA binding for the $h_{cE2}H_4R$ after expression in the presence of hydrophilic THIO indicate a ligand-induced

conformational stabilization of the already membrane-integrated $h_{cE2}H_4R$ (Schneider *et al.*, 2009).

5.4.3 [^{3}H]HA saturation binding experiments at H_4R wild-type and chimeric isoforms

Results of saturation binding experiments are shown in Table 5.2 and Fig. 5.5. Data for the hH_4R and the cH_4R were taken from Schnell *et al.* (Schnell *et al.*, 2011), where GAIP (regulator of G protein signalling 19) was additionally co-expressed, and were generated in parallel with data for chimeric H_4Rs. Co-expression of GAIP did not alter the binding properties of [^{3}H]HA to H_4Rs (data not shown) (Schneider and Seifert, 2009). Comparison of K_D values revealed no significant difference in [^{3}H]HA binding to the hH_4R, cH_4R and chimeric H_4R. The K_D value for the cH_4R is about 1.5-fold higher than K_D value for the hH_4R (Schnell *et al.*, 2011) and determined K_D values for the $h_{cNT}H_4R$, $h_{cE1}H_4R$, $h_{cE2}H_4R$ and $h_{cE3}H_4R$ were in the range between K_D values of the hH_4R and the cH_4R. The B_{max} values of the hH_4R and the $h_{cNT}H_4R$ were in the same range and the B_{max} value of the $h_{cE1}H_4R$ was even significantly higher than the B_{max} at hH_4R. In contrast, specific binding of [^{3}H]HA to the cH_4R, $h_{cE2}H_4R$ and $h_{cE3}H_4R$ significantly decreased compared to the hH_4R.

Table 5.2 [^{3}H]HA saturation binding at the hH_4R, cH_4R and four H_4R chimeras. Receptors were co-expressed in Sf9 insect cells with $G\alpha_{i2}$ and $G\beta_1\gamma_2$.

	K_D [nM]	B_{max} [pmol/mg]
hH_4R[a]	9.78 ± 0.89	1.77 ± 0.05
cH_4R[a]	15.5 ± 1.09	0.37 ± 0.01**
$h_{cNT}H_4R$	10.9 ± 0.98	2.08 ± 0.23^{+++}
$h_{cE1}H_4R$	15.2 ± 0.95	2.73 ± 0.34$^{*,+++}$
$h_{cE2}H_4R$[b]	10.8 ± 1.20	0.66 ± 0.07$^{**,\bullet\bullet,\#\#\#}$
$h_{cE3}H_4R$	15.2 ± 2.52	0.48 ± 0.04$^{**,\bullet\bullet\bullet,\#\#\#}$

In membranes, expressing the hH_4R or the cH_4R, GAIP (regulator of G protein signalling 19) was additionally co-expressed. Co-expression of GAIP does not alter the binding properties of [^{3}H]HA to H_4 receptors (see section 5.4.3). Sf9 cell membranes (100 to 200 µg protein per tube) were incubated with 0.78 to 100 nM [^{3}H]HA as described in section 5.3.5. The binding data were analyzed by non-linear regression and were best fitted to one-site (monophasic) saturation curves. Data shown are the means ± S.E.M. of three independent experiments performed in triplicate. K_D and B_{max} values were compared using one-way ANOVA, followed by Bonferroni`s multiple comparison test (B_{max} significantly different to: *hH_4R, $^+cH_4R$, $^\bullet h_{cNT}H_4R$ or $^\#h_{cE1}H_4R$; one symbol: $p < 0.05$, two symbols: $p < 0.01$, three symbols: $p < 0.001$).

[a] Data were taken from Schnell *et al.* (Schnell *et al.*, 2011) and were generated in parallel with the data on chimeric receptors to allow direct comparison.

[b] Expression in the presence of 1 µM THIO (see section 5.4.2).

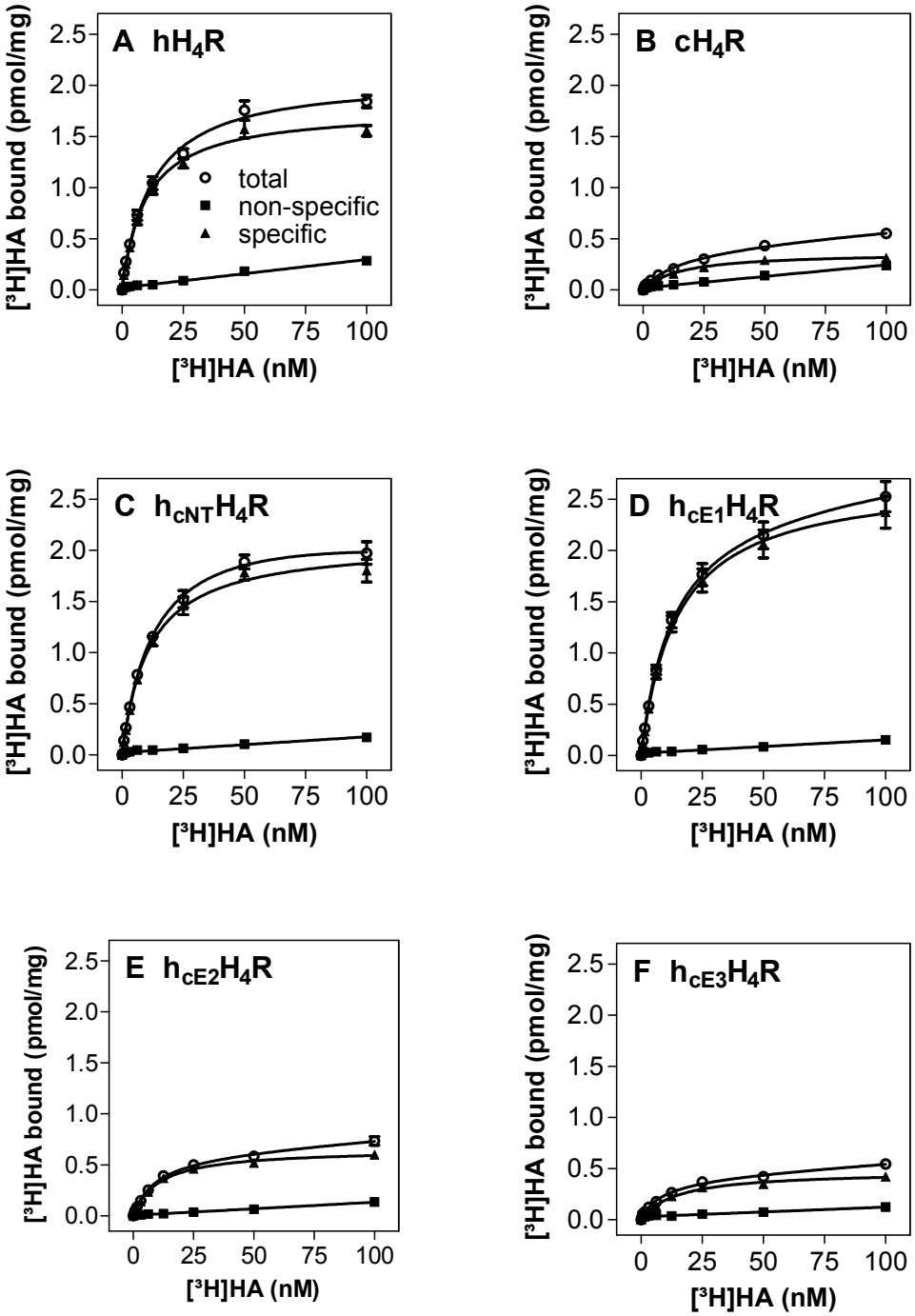

Fig. 5.5 [^{3}H]HA saturation binding curves for the hH_4R (A), cH_4R (B), $h_{cNT}H_4R$ (C), $h_{cE1}H_4R$ (D), $h_{cE2}H_4R$ (E) and $h_{cE3}H_4R$ (F). Receptors were co-expressed in Sf9 cells with $G\alpha_{i2}$ and $G\beta_1\gamma_2$. In membranes, expressing the hH_4R or the cH_4R, GAIP (regulator of G protein signalling 19) was additionally co-expressed; those data are taken from Schnell *et al.* (Schnell *et al.*, 2011) but were generated in parallel with the data on chimeric receptors. Co-expression of GAIP does not alter the binding properties of [^{3}H]HA to H_4 receptors (see section 5.4.3). The $h_{cE2}H_4R$ was expressed in the presence of 1 µM THIO (see section 5.4.2). The saturation binding experiments were performed as described in section 5.3.5, using Sf9 cell membranes (100 to 200 µg protein per tube) and [^{3}H]HA at concentrations, indicated on the abscissa. Data were analyzed by non-linear regression and were best fitted to one-site (monophasic) saturation curves. Data shown are the means ± S.E.M. of three independent experiments performed in triplicate. The open cycle (ο) shows the data for total binding, the square (■) the data for non-specific binding and the triangle (▲) the data for specific binding.

5.4.4 [^{3}H]HA competition binding experiments at H_4R wild-type and chimeric isoforms

The affinities (pK_i values) of standard H_4R ligands, determined in the [^{3}H]HA competition binding experiments, are shown in Table 5.3. HA, 5-MHA, JNJ7777120 and clozapine showed significantly lower affinity at the cH_4R than at the hH_4R, which is in accordance with recently published data (Jiang *et al.*, 2008; Lim *et al.*, 2010; Schnell *et al.*, 2011). Nevertheless, the absolute pK_i values for JNJ7777120 at the hH_4R and the cH_4R, determined in our study, differ from pK_i values, determined at the hH_4R and the cH_4R, expressed in HEK 293T cells (Lim *et al.*, 2010), although the same radioligand was used in both studies. We have no explanation for this discrepancy between data, but it should be kept in mind, that especially in case of JNJ7777120, affinity and potency in different test systems can differ greatly (for a comprehensive overview of pharmacological data regarding characterisation of JNJ7777120 in different *in vitro* test systems cf. Seifert *et al.* (2011)).

Table 5.3 Affinities of standard H_4R ligands at the hH_4R, cH_4R, $h_{cNT}H_4R$, $h_{cE1}H_4R$, $h_{cE2}H_4R$ and $h_{cE3}H_4R$, co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in Sf9 cell membranes.

	pK_i				
	HA	**5-MHA**	**THIO**	**JNJ7777120**	**clozapine**
hH_4R	7.89 ± 0.01	7.61 ± 0.06	6.94 ± 0.05	7.49 ± 0.07	6.34 ± 0.15
cH_4R	7.59 ± 0.01**	6.93 ± 0.11***	6.72 ± 0.04	6.76 ± 0.05***	3.81 ± 0.09***
$h_{cNT}H_4R$	7.86 ± 0.03^{++}	7.53 ± 0.04^{++}	7.02 ± 0.04	7.46 ± 0.06^{+++}	6.23 ± 0.02^{+++}
$h_{cE1}H_4R$	7.72 ± 0.06	7.38 ± 0.04^{+}	7.14 ± 0.06^{+}	7.25 ± 0.06^{+++}	6.18 ± 0.06^{+++}
$h_{cE2}H_4R$[a]	7.81 ± 0.01^{+}	7.27 ± 0.05	6.77 ± 0.05$^{\#}$	7.27 ± 0.03^{+++}	6.21 ± 0.06^{+++}
$h_{cE3}H_4R$	7.77 ± 0.05	7.07 ± 0.10**$^{,\bullet}$	6.74 ± 0.12$^{\#}$	7.76 ± 0.07$^{+++,\#\#\#,\phi\phi\phi}$	6.15 ± 0.07^{+++}

Competition binding was determined in Sf9 membranes (100 µg protein per tube) in the presence of 20 nM [^{3}H]HA as described in section 5.3.5. Data were analyzed by non-linear regression and were best fitted to one-site (monophasic) competition curves. Data shown are the means ± S.E.M. of three to four independent experiments performed in duplicate. pK_i values were calculated according to Cheng and Prusoff (Cheng and Prusoff, 1973) and compared using one-way ANOVA, followed by Bonferroni`s multiple comparison test (pK_i significantly different to: *hH_4R, $^{+}cH_4R$, $^{\bullet}h_{cNT}H_4R$, $^{\#}h_{cE1}H_4R$ or $^{\phi}h_{cE2}H_4R$; one symbol: $p < 0.05$, two symbols: $p < 0.01$, three symbols: $p < 0.001$).
[a] Expression in the presence of 1 µM THIO (see section 5.4.2).

The pK_i value for THIO was comparable between both wild-type receptors as already reported (Jiang *et al.*, 2008; Schnell *et al.*, 2011). Generally, the alteration of extracellular domains at the hH_4R did not significantly influence binding of examined ligands. pK_i values for HA, 5-MHA, THIO, JNJ7777120 and clozapine were in the same range at chimeric receptors ($h_{cNT}H_4R$, $h_{cE1}H_4R$, $h_{cE2}H_4R$, $h_{cE3}H_4R$) and at the wild-type hH_4R. One exception is a significantly lower affinity of 5-MHA at the $h_{cE3}H_4R$ compared to the hH_4R. Interestingly, some significantly different pK_i values were identified, when affinity of examined

ligands was compared within receptor chimeras (e.g. pK_i value for THIO at the $h_{cE1}H_4R$ and the $h_{cE2}H_4R$). This could indicate a subtle influence of individual extracellular domains onto the binding of some ligands, which remain uncovered, when pK_i values for receptor chimeras are compared solely with the wild-type hH_4R. Moreover, affinity of numerous ligands for chimeric receptors differed significantly from affinity at the wild-type cH_4R. In general, this can be explained by the similarity between the hH_4R and H_4R chimeras with respect to ligand binding.

5.4.5 Functional analysis of wild-type and chimeric H_4 receptors by steady-state GTPase activity assay

In order to study the influence of altered extracellular regions of the hH_4R on receptor activity, we determined potencies (pEC_{50} values) and efficacies (E_{max} values) of H_4R standard ligands HA, 5-MHA, THIO, JNJ7777120 and clozapine at wild-type and chimeric H_4 receptors (Table 5.4 and Fig. 5.6). In agreement with recently published data, endogenous full agonist HA had lower potency at the cH_4R than at the hH_4R (Jiang *et al.*, 2008; Schnell *et al.*, 2011). While the potency of HA at the $h_{cNT}H_4R$ and the $h_{cE1}H_4R$ was similar to the potency at the hH_4R, the potency of HA slightly (non-significantly) decreased at the $h_{cE2}H_4R$ (~2-fold) and significantly decreased at the $h_{cE3}H_4R$ (~3-fold). 5-MHA exhibited full agonistic activity at all H_4 receptors, and the same potency-pattern was observed as for the full agonist HA (comparable pEC_{50} value for the hH_4R, $h_{cNT}H_4R$ and $h_{cE1}H_4R$, slightly decreased pEC_{50} at the $h_{cE2}H_4R$ and significantly decreased pEC_{50} at the $h_{cE3}H_4R$ and the cH_4R). At the hH_4R and chimeric H_4Rs clozapine acted as partial agonist, but showed no stimulation of GTPase activity at cH_4R. Interestingly, the potency of clozapine at the $h_{cE1}H_4R$ and the $h_{cE3}H_4R$ was by more than 0.5 log units lower than at the $h_{cNT}H_4R$, although neither of chimeric H_4 receptors significantly differs from the hH_4R in pEC_{50} value for clozapine.

Additionally, we examined two ligands with inverse agonistic activity at the hH_4R. THIO acts at the hH_4R as almost full inverse agonist and JNJ7777120 as weak inverse agonist (Schneider *et al.*, 2009). In accordance with recently published data (Schnell *et al.*, 2011), we observed a shift from inverse agonistic activity at the hH_4R to agonistic activity at the cH_4R for both THIO and JNJ7777120 (Table 5.4). The mode of action and the E_{max} values for THIO and JNJ-7777120 were not altered at the $h_{cNT}H_4R$ and the $h_{cE1}H_4R$ in comparison with the hH_4R. However, a decrease in inverse agonistic activity of THIO was observed in the series $hH_4R > h_{cE2}H_4R > h_{cE3}H_4R$. Moreover, JNJ7777120 acted as partial agonist at the $h_{cE2}H_4R$ and the $h_{cE3}H_4R$, but with significantly lower efficacy than at the cH_4R.

Table 5.4 Potencies and efficacies of standard H_4R ligands at the hH_4R, cH_4R, $h_{cNT}H_4R$, $h_{cE1}H_4R$, $h_{cE2}H_4R$ and $h_{cE3}H_4R$, co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in Sf9 cell membranes.

	HA		5-MHA		THIO		JNJ7777120		clozapine	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}
hH_4R	7.60 ± 0.12	1.00	7.11 ± 0.05	1.03 ± 0.06	6.87 ± 0.14	-0.87 ± 0.08	8.25 ± 0.17	-0.16 ± 0.01	6.02 ± 0.09	0.64 ± 0.06
cH_4R	6.76 ± 0.04 ***	1.00	6.13 ± 0.02 ***	0.93 ± 0.01	6.40 ± 0.10	0.28 ± 0.04 ***	6.20 ± 0.17 ***	0.66 ± 0.03 ***	no effect	no effect
$h_{cNT}H_4R$	7.55 ± 0.07 +++	1.00	7.29 ± 0.16 +++	1.02 ± 0.06	6.99 ± 0.09	-0.93 ± 0.06 +++	7.12 ± 0.05 ***,++	-0.20 ± 0.04 +++	6.41 ± 0.15	0.65 ± 0.05
$h_{cE1}H_4R$	7.41 ± 0.13 ++	1.00	7.20 ± 0.10 +++	1.04 ± 0.06	6.60 ± 0.12	-0.85 ± 0.03 +++	n.d.[b]	-0.18 ± 0.04 +++	5.83 ± 0.10 •	0.63 ± 0.10
$h_{cE2}H_4R$[a]	7.28 ± 0.03 +	1.00	6.78 ± 0.07 +	1.03 ± 0.02	6.78 ± 0.06	-0.43 ± 0.01 ***,+++,•••,##	6.98 ± 0.07 ***,+	0.23 ± 0.02 ***,+++,•••,###	6.20 ± 0.03	0.73 ± 0.03
$h_{cE3}H_4R$	7.09 ± 0.04 *	1.00	6.46 ± 0.14 *,••,##	1.08 ± 0.06	7.00 ± 0.25	-0.27± 0.02 ***,+++,•••,###	7.24 ± 0.08 **,++	0.21 ± 0.05 ***,+++,•••,###	5.88 ± 0.06 •	0.60 ± 0.01

GTPase activity was determined in Sf9 membranes (10 to 20 µg protein per tube) as described in section 5.3.6. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are the means ± S.E.M. of three to five independent experiments performed in duplicate or triplicate. The efficacy (E_{max}) of HA was set to 1.00 and the efficacies of other ligands were referred to this value. pEC_{50} and E_{max} values were compared using one-way ANOVA, followed by Bonferroni`s multiple comparison test (pEC_{50} or E_{max} significantly different to: *hH_4R, $^{+}cH_4R$, $^{\bullet}h_{cNT}H_4R$, $^{\#}h_{cE1}H_4R$; one symbol: $p < 0.05$, two symbols: $p < 0.01$, three symbols: $p < 0.001$).

[a] Expression in the presence of 1 µM THIO (see section 5.4.2).

[b] n.d., not determined. Because of the small inhibitory effect at the $h_{cE1}H_4R$, pEC_{50} value for JNJ7777120 could not be determined. E_{max} value was assessed using 10 or 100 µM JNJ7777120.

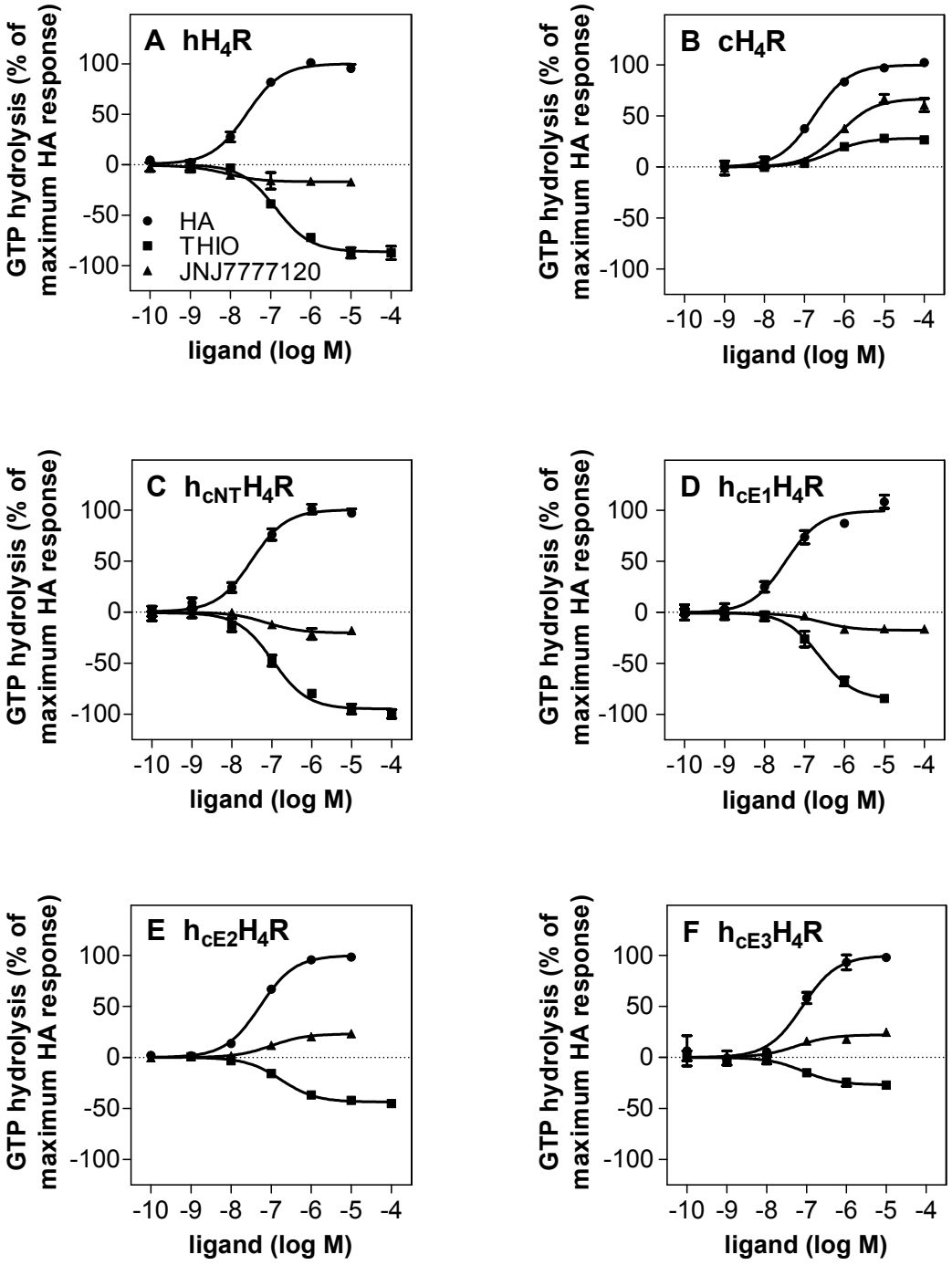

Fig. 5.6 Concentration-response curves for HA (●), THIO (■) and JNJ7777120 (▲) at the hH_4R (A), cH_4R (B), $h_{cNT}H_4R$ (C), $h_{cE1}H_4R$ (D), $h_{cE2}H_4R$ (E) and $h_{cE3}H_4R$ (F), determined in steady-state GTPase activity assay. Receptors were co-expressed in Sf9 cells with $G\alpha_{i2}$ and $G\beta_1\gamma_2$. The $h_{cE2}H_4R$ was expressed in the presence of 1 µM THIO (see section 5.4.2). GTP hydrolysis in Sf9 membranes was measured as described in section 5.3.6. Reaction mixtures contained Sf9 membranes (10 to 20 µg protein per tube) and ligands at concentrations indicated on the abscissa. Data shown are means ± S.E.M. of at least three independent experiments performed in duplicate or triplicate. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration-response curves.

5.4.6 Functional analysis of the hH_4R-$G\alpha_{i2}$ and the $h_{cE3}H_4R$-$G\alpha_{i2}$ fusion proteins by steady-state GTPase assay

When co-expression system is used for the pharmacological characterization of one GPCR, we cannot assure a constant receptor/G-protein ratio (Schneider and Seifert, 2010). Consequently, coupling efficiency of receptor to G-protein might be influenced. In contrast, when the C-terminus of the GPCR is fused to the N-terminus of the G-protein, close proximity of both interaction partners enables increased interaction efficiency. In addition, fusion proteins assure characterization of receptor/G-protein coupling in a system with defined 1:1 stoichiometry (Schneider and Seifert, 2010).

When we consider the data from functional assays (Table 5.4), the $h_{cE3}H_4R$ differed in pharmacological profile from the hH_4R the most among all chimeric receptors. Therefore, we decided to analyze the influence of potentially unequal H_4 receptor/$G\alpha_{i2}$ ratio on the different pharmacological characteristics of the $h_{cE3}H_4R$ and the hH_4R. For this reason, fusion proteins hH_4R-$G\alpha_{i2}$ and $h_{cE3}H_4R$-$G\alpha_{i2}$ were co-expressed with $G\beta_1\gamma_2$ in Sf9 insect cells and characterized by steady-state GTPase assay (Table 5.5). Afterwards, potencies and efficacies of HA, 5-MHA and THIO were compared to the corresponding parameters from co-expression system (hH_4R or $h_{cE3}H_4R$ + $G\alpha_{i2}$ + $G\beta_1\gamma_2$), shown in Table 5.4. Statistical analysis revealed no significant alteration of potency for HA, 5-MHA and THIO neither for the hH_4R nor for the $h_{cE3}H_4R$, when they were fused to $G\alpha_{i2}$ (Table 5.5). Interestingly, the efficacy of the inverse agonist THIO markedly (1.9-fold) increased at the fusion protein hH_4R-$G\alpha_{i2}$, indicating an increased constitutive GTPase activity related to the total ligand-regulated GTPase activity. This can be explained by the stabilizing effect of $G\alpha_{i2}$ on hH_4R active conformation in a fusion protein, where interaction partners are in close proximity to each other (Schneider *et al.*, 2009). In contrast, the efficacy of the inverse agonist THIO was not significantly increased at the $h_{cE3}H_4R$-$G\alpha_{i2}$ compared to co-expression system. Thus, very low THIO-sensitive constitutive GTPase activity of the $h_{cE3}H_4R$ relative to the total ligand-regulated GTPase activity is not due to inefficient interaction with $G\alpha_{i2}$. In summary, the altered pharmacological profile of the $h_{cE3}H_4R$ compared to the hH_4R is not due to the unequal receptor/G-protein ratio, but due to differences in the E3-loop.

Table 5.5 Potencies and efficacies of HA, 5-MHA and THIO at the hH_4R-$G\alpha_{i2}$ and $h_{cE3}H_4R$-$G\alpha_{i2}$ fusion proteins, co-expressed with $G\beta_1\gamma_2$ in Sf9 cell membranes.

Compound	hH_4R-$G\alpha_{i2}$		$h_{cE3}H_4R$-$G\alpha_{i2}$	
	pEC_{50} [a]	E_{max} [b]	pEC_{50} [a]	E_{max} [b]
HA	7.53 ± 0.05	1.00	7.05 ± 0.01	1.00
5-MHA	7.33 ± 0.08	1.19 ± 0.14	6.42 ± 0.01	0.98 ± 0.05
THIO	6.81 ± 0.07	-1.67 ± 0.30*	6.84 ± 0.14	-0.37± 0.03

GTPase activity was determined in Sf9 membranes (10 to 20 µg protein per tube) as described in section 5.3.6. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are the means ± S.E.M. of three to four independent experiments performed in duplicate or triplicate. The efficacy (E_{max}) of HA was set to 1.00 and the efficacies of other ligands were referred to this value. pEC_{50} and E_{max} values for HA, 5-MHA and THIO at the hH_4R-$G\alpha_{i2}$ fusion protein were compared to corresponding parameters at the hH_4R (data from Table 5.4) and pEC_{50} and E_{max} values for HA, 5-MHA and THIO at the $h_{cE3}H_4R$-$G\alpha_{i2}$ fusion protein were compared to corresponding parameters at the $h_{cE3}H_4R$ (data from Table 5.4) using unpaired two-tailed t test (95 % confidence interval).

[a] Comparison with the pEC_{50} at the hH_4R; no significant difference.

[b] Comparison with the E_{max} at the hH_4R; *, $p < 0.05$.

[c] Comparison with the pEC_{50} at the $h_{cE3}H_4R$; no significant difference.

[d] Comparison with the E_{max} at the $h_{cE3}H_4R$; no significant difference.

5.4.7 Binding of JNJ7777120 to the hH_4R, $h_{cE2}H_4R$, $h_{cE3}H_4R$ and cH_4R

Since JNJ7777120 acts as inverse agonist at the hH_4R, but as partial agonist at the $h_{cE2}H_4R$, $h_{cE3}H_4R$ and cH_4R, JNJ7777120 was studied within the inactive model of the hH_4R and within the active state models of the $h_{cE2}H_4R$, $h_{cE3}H_4R$ and cH_4R. The MD simulations revealed stable binding modes of JNJ7777120 at the hH_4R, $h_{cE2}H_4R$, $h_{cE3}H_4R$ and cH_4R. However, there were significant differences between the binding of JNJ7777120 at the hH_4R on the one hand and at the $h_{cE2}H_4R$, $h_{cE3}H_4R$ and cH_4R on the other hand.

A MD snapshot of JNJ7777120 in the binding pocket of the inactive hH_4R is given in Fig. 5.7A. The basic amine moiety of JNJ7777120 establishes a stable electrostatic interaction with the highly conserved $Asp^{3.32}$. The indole moiety of JNJ7777120 is embedded in an aromatic pocket, established by $Tyr^{3.33}$, $Trp^{6.48}$ and $Tyr^{6.51}$. The $Trp^{6.48}$, discussed to be involved in the rotamer toggle switch during receptor activation (Crocker *et al.*, 2006) is stabilized in a more vertical conformation, corresponding to the inactive state.

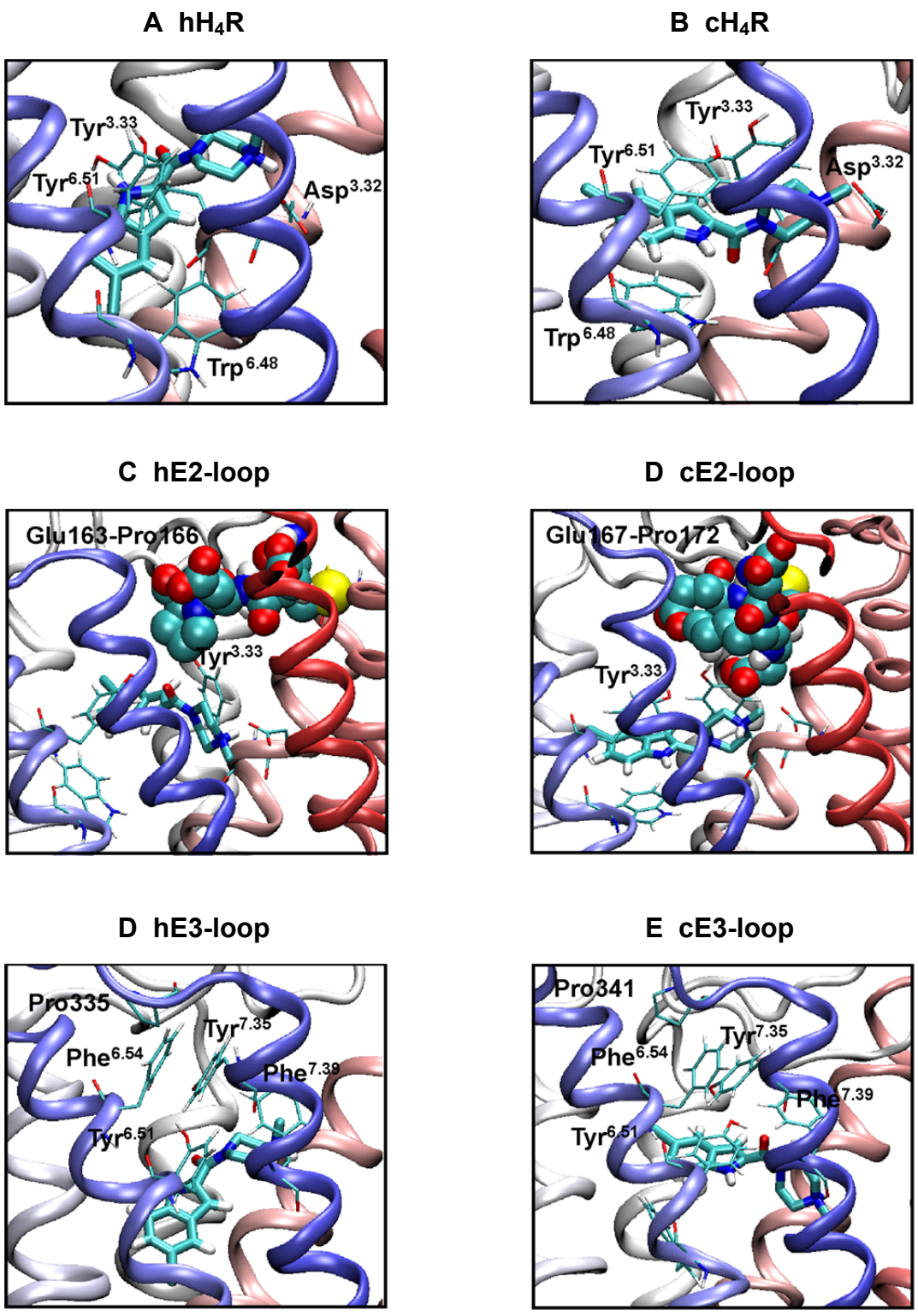

Fig. 5.7 Binding mode of JNJ777120 at the hH_4R and the cH_4R and influence of E2- and E3-loop onto the binding pocket. (**A**) Binding mode of JNJ7777120 at the hH_4R; (**B**) binding mode of JNJ7777120 at the cH_4R; (**C**) influence of the human E2-loop onto the binding pocket (amino acids Glu163-Pro166 of the E2-loop presented as van der Waals spheres); (**D**) influence of the canine E2-loop onto the binding pocket (amino acids Glu167-Pro172 of the E2-loop presented as van der Waals spheres); (**E**) influence of the human E3-loop onto the binding pocket; (**F**) influence of the canine E3-loop onto the binding pocket. Molecular modeling studies were performed as described in section 5.3.7.

In contrast, at the $h_{cE2}H_4R$, $h_{cE3}H_4R$ and cH_4R, the JNJ7777120 stabilizes the active receptor conformation. The MD simulations revealed no significant differences for the binding mode of JNJ7777120 at the $h_{cE2}H_4R$, $h_{cE3}H_4R$ and cH_4R. Representative for the $h_{cE2}H_4R$, $h_{cE3}H_4R$ and cH_4R, the binding mode of JNJ7777120 at the cH_4R is given in Fig. 5.7B. As already observed at the hH_4R, the positively charged amine moiety of JNJ7777120 interacts electrostatically with the highly conserved $Asp^{3.32}$. The indole moiety of JNJ7777120 is embedded in an aromatic pocket, established by $Tyr^{3.33}$, $Trp^{6.48}$ and $Tyr^{6.51}$. In contrast to the binding mode of JNJ7777120 at the inactive hH_4R, the $Trp^{6.48}$ shows a more horizontal conformation, corresponding to the active receptor state. The orientation of the indole moiety of JNJ7777120 restrains the $Trp^{6.48}$ in its active conformation, due to a stacked aromatic interaction.

The differences between human and canine E2-loop are shown in Fig. 5.7C (human E2-loop) and Fig. 5.7D (canine E2-loop). For the human E2-loop, the amino acids Glu163, Cys164, Glu165 and mainly Pro166 are in close contact to $Tyr^{3.33}$. The interaction between this part of the human E2-loop and $Tyr^{3.33}$ forces the $Tyr^{3.33}$ away from the binding pocket (Fig. 5.7C). The corresponding interaction is quite different for the canine E2-loop. Here, Glu167, Lys168, Asp169, Cys170, Glu171 and Pro172 are in close contact to $Tyr^{3.33}$. This more polar interaction surface forces the $Tyr^{3.33}$ in direction of JNJ7777120 in the binding pocket, enabling a stronger interaction between JNJ7777120 and $Tyr^{3.33}$. Furthermore, this conformation of $Tyr^{3.33}$ stabilizes the ligand-receptor complex in the active state.

The different influence of human and canine E3-loop is shown in Fig. 5.7E (human E3-loop) and Fig. 5.7F (canine E3-loop). The E3-loop is in close contact to an aromatic channel, passing from the extracellular part into the binding pocket. This channel is formed by $Phe^{6.54}$, $Tyr^{6.51}$, $Tyr^{7.35}$ and $Phe^{7.39}$ at the hH_4R as well as at the cH_4R. A proline (Pro335 at the hH_4R, Pro341 at the cH_4R) is in close contact to the entry of this aromatic channel, established by $Phe^{6.54}$ and $Tyr^{7.35}$. Nevertheless, the conformations of the E3-loop differ between the hH_4R and the cH_4R. Thus, it is suggested that the conformation of the E3-loop, especially, the orientation of the proline (Pro335 at the hH_4R, Pro341 at the cH_4R), is responsible for the different conformations of the aromatic channel between TM VI and TM VII, leading from the extracellular part into the binding pocket.

Additionally, it should be noted that JNJ7777120 can be docked into both, the inactive and the active hH_4R (Seifert *et al.*, 2011). Since the differences between the binding pockets of the hH_4R, the cH_4R and the chimeras are not very large, it is reasonable to assume that JNJ7777120 can be docked in inactive and active models of the cH_4R and the chimeras. However, the docking studies would not give information about the thermodynamic stability of the corresponding ligand-receptor complexes. Therefore, molecular dynamic

simulations in combination with calculation of Gibbs energies would be appreciated. Unfortunately, up to now, a reliable prediction of Gibbs energies is not possible.

5.5 Discussion

Numerous H_4R species were pharmacologically characterized in various recombinant test systems (Liu *et al.*, 2001; Jiang *et al.*, 2008; Lim *et al.*, 2008; Lim *et al.*, 2010; Schnell *et al.*, 2011). The aforementioned studies revealed differences in binding affinity, potency and efficacy of ligands at H_4R species orthologs and sometimes even discrepancies in mode of action (agonistic, antagonistic or inverse agonistic activity) (Schnell *et al.*, 2011). Searching for structural determinants, responsible for these differing pharmacological characteristics, we focused on four extracellular regions of the H_4R, i.e. N-terminus and three extracellular loops. So far, relatively little attention has been paid to extracellular regions of biogenic amine GPCRs, although the number of studies is increasing (Shi and Javitch, 2002; Lawson and Wheatley, 2004; Peeters *et al.*, 2011b). One of the reasons for the insufficient evaluation of extracellular domains is that, based on crystal structures, only a snapshot-conformation of the highly flexible extracellular part of receptors is available. Thus, information about dynamics of the extracellular part is lost. However, it is possible to address the dynamics of the extracellular part of GPCRs by MD simulations. Pharmacological characterization of chimeric receptors in combination with MD simulation is a very useful approach. In our study, human/canine chimeric H_4Rs were used to assess the importance of extracellular domains for H_4R species differences. The results may contribute to broaden the knowledge about the functional role of extracellular regions of aminergic GPCRs.

5.5.1 Ligand-induced stabilization of the $h_{cE2}H_4R$ during expression

In order to evaluate the potential involvement of single extracellular regions in differences between hH_4R and cH_4R pharmacology, four human/canine H_4R chimeras were constructed ($h_{cNT}H_4R$, $h_{cE1}H_4R$, $h_{cE2}H_4R$ and $h_{cE3}H_4R$). Among them, the chimeric receptor $h_{cE2}H_4R$ showed high structural instability. Presumably, additional 6 amino acids in the E2-loop of the $h_{cE2}H_4R$ and a few shifts in charge of amino acids compared to the hH_4R are responsible for disruption of receptor-stabilizing constraints. Nevertheless, further pharmacological analysis of the $h_{cE2}H_4R$ became feasible because we could stabilize the $h_{cE2}H_4R$ by expression in the presence of the inverse agonist THIO. Structural instability of GPCRs and their stabilization by ligands has been observed repeatedly (Gether *et al.*, 1997; Pauwels and Tardif, 2002; Roth *et al.*, 2008; Schneider *et al.*, 2009).

It is interesting that expression of the $h_{cE2}H_4R$ with HA or THIO increases the number of high-affinity [^{3}H]HA binding sites to the same extent, whereas the improvement of signal-to-noise ratio in GTPase assay was superior in case of expression in the presence of THIO. Both hydrophilic ligands can interact with receptors, which are already present on the membrane surface. After binding of ligands during expression, interactions in the receptor structure and those of receptor with environment are rearranged, resulting in increased stability of inherently unstable receptors. Obviously, the $h_{cE2}H_4R$ adopts similar receptor conformation (similar equilibrium between active and inactive state) after washing out of HA or THIO, which is equally recognized by [^{3}H]HA. But still, stabilization of the $h_{cE2}H_4R$ with THIO presumably rearranges additional important interactions and allows receptors to more intensively undergo transitions between active and inactive state after incubation with an agonist or inverse agonist, respectively. Superior effects of the inverse agonist ICI 118,551 ((2*R**,3*S**)-3-(isopropylamino)-1-(7-methyl-2,3-dihydro-1*H*-inden-4-yloxy)butan-2-ol) *vs.* the agonist isoproterenol were already observed in denaturation studies with a constitutively active and structurally unstable mutant of the β_2-adrenergic receptor (Gether *et al.*, 1997).

Additionally, the question arose whether the pharmacological characteristics of the $h_{cE2}H_4R$ change due to expression in the presence of THIO. Because of the low signal-to-noise ratio at the $h_{cE2}H_4R$ expressed under control conditions, comparison of efficacies and potencies of HA and THIO with those at the $h_{cE2}H_4R$, expressed in the presence of THIO, was impossible. Nevertheless, expression with THIO did not influence pEC_{50} and E_{max} values for HA and THIO at the hH_4R, determined in steady-state GTPase assay (data not shown). Additionally, the expression of wild-type histamine H_2 receptor in the presence of ranitidine did neither significantly affect the binding of [^{125}I]aminopotentidine nor change pEC_{50} and E_{max} values of the histamine-induced cAMP production (Alewijnse *et al.*, 2000). Furthermore, K_D value of [^{3}H]RX821002 (2-(2-methoxy-2,3-dihydrobenzo[b][1,4]dioxin-2-yl)-4,5-dihydro-1*H*-imidazole) binding to mutated α_{2A} adrenergic receptor (Thr373Lys α_{2A}AR) was neither affected by the expression of receptor in the presence of inverse agonist nor in the presence of antagonist or agonist (Pauwels and Tardif, 2002). Based on these data, indicating unaltered receptor properties after expression with various ligands, we decided to express the $h_{cE2}H_4R$ in the presence of 1 µM THIO to allow its further pharmacological characterization.

5.5.2 Irrelevance of the N-terminus and E1-loop for differences between the hH_4R and the cH_4R

The N-terminus of aminergic GPCRs is generally considered to be a pharmacologically inert element. One notable exception is the N-terminus of the histamine H_1 receptor, where the alteration of hydrogen bond network between N-terminus and E2-loop

influenced binding of histaprodifens (Strasser *et al.*, 2008b). Also mutations of two amino acids in the E1-loop of adenosine A_{2B} receptor lead to changes in constitutive activity and potency of agonists (Peeters *et al.*, 2011a). Our study revealed that the N-terminus and E1-loop are not responsible for different characteristics of the hH_4R and the cH_4R. One reason could be a relative high amino acid homology for N-terminus and E1-loop between the hH_4R and the cH_4R. Besides the intracellular C-terminus, extracellular regions are generally the most variable elements of GPCRs. Additionally, only a few shifts in charge were caused by the generation of $h_{cNT}H_4R$ (Asp3→Ala3) and $h_{cE1}H_4R$ mutants (Lys84→Asn84, Glu85→Lys85). Charged residues in E1-loop were crucial for activation of some other GPCRs (Peeters *et al.*, 2011b).

5.5.3 Binding characteristics of the $h_{cE2}H_4R$ and the $h_{cE3}H_4R$

Several studies report about versatile roles of E2-loop in aminergic GPCRs, like "gatekeeping" of the binding crevice, recognition of ligands and allosteric modulation (Peeters *et al.*, 2011b). Importance of E2-loop for receptor subtype and species selectivity was reported amongst others for α_1-adrenergic receptor and 5-HT_{1D} receptor (Shi and Javitch, 2002). Also reports about involvement of E3-loop in ligand recognition process and activation mechanism of aminergic GPCRs are increasing (Lawson and Wheatley, 2004; Peeters *et al.*, 2011b).

We assessed the importance of E2- and E3-loop of H_4R for ligand binding and receptor activation, using chimeric receptors $h_{cE2}H_4R$ and $h_{cE3}H_4R$. Worthy of note is lower species sequence homology for E2-loop (48 %) and E3-loop (20 %) than for N-terminus and E1-loop. Binding experiments revealed no major impact of E2- and E3-loop on affinity of examined ligands. In contrast, a single amino acid (Phe169) in E2-loop is responsible for difference in agonist binding to the human and mouse H_4R (Lim *et al.*, 2008). Phe169 is present at equivalent position at the hH_4R and at the cH_4R.

A striking discrepancy was found between B_{max} values from [^{3}HA] radioligand binding studies and relative density of bands, obtained by immunoblotting when the hH_4R was compared with the $h_{cE2}H_4R$ and the $h_{cE3}H_4R$. B_{max} for [^{3}H]HA binding at the $h_{cE2}H_4R$ and the $h_{cE3}H_4R$ is 2.7- and 3.7-fold lower than at the hH_4R, which is not reflected in relative densities of bands from immunoblot studies. Proteolysis of H_4R chimeras does not account for the discrepancies since no bands with lower mass than the predicted receptor mass were detected. Hence, there are two plausible explanations for this phenomenon. Firstly, membrane integrated $h_{cE2}H_4R$ and $h_{cE3}H_4R$ are structurally unstable and therefore unable to bind [^{3}H]HA (Schneider *et al.*, 2009). Secondly, the proportion of low-affinity binding sites for the agonist [^{3}H]HA is increased in case of E2- and E3-loop mutant. Unfortunately, in binding experiments with [^{3}H]HA, low-affinity binding sites cannot be easily detected. By contrast, in

functional GTPase experiments, low-affinity ligand-receptor interactions can be readily recognized (Wenzel-Seifert *et al.*, 1999), but for H_4R chimeras, we did not obtain evidence for such low-affinity interaction.

Generally, the best way to distinguish between high- and low-affinity agonist binding sites would be an additional application of radiolabeled antagonist. In competition studies with a radiolabeled antagonist, low-affinity agonist binding sites can be readily detected (Seifert *et al.*, 1998a; Seifert *et al.*, 1998b; Seifert *et al.*, 2001). [^{3}H]JNJ7777120 was previously used as H_4R antagonist radioligand (Lim *et al.*, 2005). In our study, [^{3}H]JNJ7777120 cannot be used because JNJ7777120 acts as partial agonist at the $h_{cE2}H_4R$ and the $h_{cE3}H_4R$. An alternative approach to investigate high- and low-affinity binding sites are studies with an non-hydrolysable analogue of GTP, guanosine 5'-[γ-thio]triphosphate (GTPγS). Binding of GTPγS to G-protein causes uncoupling of receptor and G-protein, resulting in a reduction of high-affinity binding sites (De Lean *et al.*, 1980). But even here, the problems occur in case of the H_4R. High-affinity receptor state of the hH_4R is insensitive to GTPγS (Schneider *et al.*, 2009).

5.5.4 Involvement of the E2- and E3-loop in H_4R activation process

For the H_4R, we provide for the first time evidence about the importance of E2- and E3-loop with respect to receptor activity. Specifically, our study revealed a crucial role of E2- and E3-loop in the paradoxical agonistic activity of THIO and JNJ7777120 at the cH_4R (Schnell *et al.*, 2011). Efficacy of both ligands at the $h_{cE2}H_4R$ and the $h_{cE3}H_4R$ is substantially shifted toward efficacy at the cH_4R. By analysis of receptor-$G\alpha_{i2}$ fusion proteins we could unequivocally demonstrate that this effect is not due to insufficient coupling of chimeric H_4Rs to $G\alpha_{i2}$.

THIO exhibits strong inverse agonistic activity at the highly constitutive active hH_4R (Schneider *et al.*, 2009), which is reduced in case of the $h_{cE2}H_4R$ and the $h_{cE3}H_4R$. At the less constitutively active cH_4R, THIO elevates GTP hydrolysis in Sf9 insect cell membranes (Schnell *et al.*, 2011). Given that the affinity of THIO to both species as same as to the $h_{cE2}H_4R$ and the $h_{cE3}H_4R$ is comparable, the cause of variable THIO efficacy profile lies in a conformational change of receptor after binding of THIO. Here, different constitutive activity of receptors could be relevant. Altered E2-loop and E3-loop at the $h_{cE2}H_4R$ and the $h_{cE3}H_4R$, respectively, reduce pronounced constitutive activity of the wild-type hH_4R and consequently equilibrium between active (R*) and inactive state (R) is partially shifted toward the inactive state. Therefore, after binding of THIO, the equilibrium R*/R cannot be shifted toward the R state to the same extent as in the case of the hH_4R (regarded relative to basal GTPase activity). Furthermore, the basal GTPase activity at the cH_4R is already so low that similar R*/R equilibrium as at the hH_4R after binding of THIO is seen as agonism (Kenakin, 2007;

Schnell *et al.*, 2011). All in all, we suggest the E2- and E3-loop to be involved in fine tuning of constitutive activity of H_4Rs.

Based on the efficacy profile of JNJ7777120 at the hH_4R, $h_{cE2}H_4R$, $h_{cE3}H_4R$ and cH_4R, we also suggest that the E2- and E3-loops are involved in inverse or partial agonistic behaviour of JNJ7777120 at H_4Rs. Since the exchange of E2-loop or E3-loop at the hH_4R for the corresponding canine loops does not lead to a complete shift of efficacy toward the cH_4R, the inverse/partial agonistic behaviour of JNJ7777120 cannot be attributed to these moieties alone. Additional differences between the sequences of the hH_4R and the cH_4R might play a role as well as a co-operative action of E2- and E3-loop. The molecular modelling studies also suggest that E2- and E3-loop affect the pharmacology of JNJ7777120 at the H_4R independently of each other.

The segment of the E2-loop starting from the cysteine (Cys164 at the hH_4R, Cys170 at the cH_4R) in direction to TM V exhibits the same length at the hH_4R and the cH_4R. The cysteine (Cys164 at the hH_4R, Cys170 at the cH_4R) establishes a disulfide bridge to $Cys^{3.25}$. Thus, the position of this part of the E2-loop is restrained and no significant differences in conformation between the hH_4R and the cH_4R were observed within the MD simulations. In contrast, there is a significant difference with regard to length of the segment of the E2-loop from TM IV to Cys164 at the hH_4R and Cys170 at the cH_4R, respectively. Thus, completely different conformations of this part of the E2-loop were observed during the MD simulations. Consequently, the contact surfaces of E2-loop to $Tyr^{3.33}$ are completely different, on the one hand with regard to volume, on the other hand with regard to surface properties. This has strong impact onto the conformation of $Tyr^{3.33}$, which is in close contact to the bound JNJ7777120. In case of the cH_4R, $Tyr^{3.33}$ is shifted more towards the binding pocket, compared to the hH_4R. Thus, at the cH_4R or the $h_{cE2}H_4R$ JNJ7777120 bound in the active receptor conformation is preferred.

There are no differences in length of human and canine E3-loop, but significant differences in amino acid sequence. Only a tyrosine (Tyr329 at the hH_4R, Tyr335 at the cH_4R) and a proline (Pro335 at the hH_4R, Pro341 at the cH_4R) are found in the E3-loops of both H_4R orthologs. The MD simulations revealed differences in conformation of E3-loop between the hH_4R and the cH_4R. Additionally, the proline (Pro335 at the hH_4R, Pro341 at the cH_4R) is in close contact to an aromatic channel, established by TM VI and TM VII, leading from the extracellular area into the binding pocket. There, $Tyr^{6.51}$ interacts with the bound JNJ7777120. Thus, the E3-loop may have an indirect effect onto the conformation of $Tyr^{6.51}$ and in this way on the binding mode of JNJ7777120.

In general, molecular modelling studies enable a deeper insight into ligand-GPCR interactions on a molecular level. However, in contrast to the transmembrane domains, the loops are highly flexible, rendering the modelling of loops much more challenging than

modelling of transmembrane domains. Nonetheless, molecular modelling studies, especially MD simulations, can provide useful hints with regard to the influence of the extracellular loops on ligand binding. Still, it is not clear, whether single amino acids in the region of E2- and E3-loop or the E2- and E3-loops as a whole are responsible for the surprising efficacy profile of JNJ7777120 at the hH_4R and the cH_4R.

5.5.5 Conclusions

The present study revealed for the first time an involvement of E2- and E3-loop in the activation process of the H_4Rs, especially with regard to the surprising efficacy profile of JNJ7777120 at the hH_4R and the cH_4R. MD simulations provide important suggestions about the influence of E2- and E3-loops onto the inverse/partial agonistic behaviour of JNJ7777120 at the hH_4R and the cH_4R on molecular level. Besides, the modelling studies suggest an independent influence of E2-loop and E3-loop onto the binding of JNJ7777120. Thus, this work is broadening our knowledge about structural determinants responsible for the species-selective efficacy profile of JNJ7777120 at H_4Rs.

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Chapter 6

Summary/Zusammenfassung

6.1 Summary

About one third of the drugs currently available on the market address cell membrane-integrated G protein-coupled receptors (GPCRs). As such, the histamine H_2 receptor (H_2R) and the β_2-adrenergic receptor (β_2AR) have been successfully targeted in the treatment of frequently occurring gastric disorders and asthma, respectively. The novel histaminergic GPCR, the histamine H_4 receptor (H_4R), is currently in the focus of clinical trials as a promising target in the therapy of asthma and allergic rhinitis. However, being involved in many (patho)physiological functions, the H_2R, H_4R and β_2AR gain great interest in academia and industry because of their potential to both, enable novel and to improve established therapeutic interventions.

The first aim of this thesis was the dissection and comparison of the H_2R- and β_2AR-mediated effects on isolated human neutrophil granulocytes. Activation of both receptors on neutrophils results in an inhibition of inflammatory responses such as superoxide anion ($O_2^{\bullet-}$) production, which could be therapeutically exploited. Although both, the H_2R and the β_2AR, preferentially couple to G_s proteins, a distinct behavior was observed in respect of their influence on cyclic adenosine 3',5'-monophosphate (cAMP) accumulation and $O_2^{\bullet-}$ production in neutrophils and a discrepancy with literature data, obtained in recombinant test systems, was noticed. In addition, ligand-directed signaling was detected, indicative of the concept of functional selectivity and suggesting that signaling events between the cAMP accumulation and the $O_2^{\bullet-}$ production are much more complex than assumed so far. Moreover, untypical behavior of H_2R antagonists on neutrophils does not fit to the conventional understanding of antagonist/GPCR interaction. Possible reasons for described observations are discussed, although more detailed analyses of the signaling events in neutrophils are needed to mechanistically explain the divergence between the H_2R and the β_2AR and to better understand the multiplicity of GPCR-mediated signaling.

The second aim of the present work was to characterize N^G-acylated hetarylpropylguanidines, highly potent (partial) human H_2R-agonists in Sf9 insect cell membranes, in physiologically more relevant human neutrophil granulocytes. In general, new H_2R agonists are developed as valuable pharmacological tools and are regarded as novel drug candidates, e.g. in the therapy of acute myeloid leukemia. In contrast to histamine, the $O_2^{\bullet-}$ production by activated neutrophils was not affected by bivalent acylguanidines, whereas a monovalent ligand reduced $O_2^{\bullet-}$ production in a H_2R-independent way. However, the ligands of interest stimulated cAMP accumulation in an H_2R-dependent manner, albeit with drastically diminished agonistic activity compared with that in the respective recombinant test system. These results point to a drawback of acylguanidines, e.g. their amphiphilic character, and suggest that their drug-likeness needs to be further improved. Moreover, a contribution to the understanding of molecular interactions between the H_2R and N^G-acylated

hetarylpropylguanidines was made. Mutagenesis studies revealed a rather marginal contribution of the extracelluar N-terminus to the species-selective profile of acylguanidines at the human H_2R and the guinea pig H_2R.

Preclinical investigation of therapeutically interesting compounds in translational animal models is indispensable in drug research and development. Thus, identification and understanding of substantial pharmacological differences between H_4R species orthologs at the molecular level is essential. In the third part of this thesis, the importance of extracellular regions for the species-specific pharmacology of the H_4R was evaluated, using chimeric receptor approach and Sf9 insect cells as expression system. This study indicates that the N-terminus and the first extracellular loop are not responsible for the species differences between human and canine H_4Rs. On the contrary, for the first time evidence is provided that the second and the third extracellular loop are involved in the H_4R activation process. Mutagenesis studies revealed that low amino acid sequence homologies of the latter two loops contribute to the divergence between the inverse agonistic activity of JNJ7777120 (1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine) at the human and partial agonistic activity at the canine H_4R, reported previously. Using a molecular dynamic simulation approach, suggestions about the involvement of the second and third extracellular loops in the inverse/partial agonistic behavior of JNJ7777120 are made at the molecular level.

Taken together, this work broadens our knowledge about the therapeutically relevant H_2R, β_2AR and H_4R at the cellular and/or molecular level.

6.2 Zusammenfassung

Ein Drittel der derzeit auf dem Markt befindlichen Arzneimittel bindet an G Protein-gekoppelte Rezeptoren (GPCR). Drei Vertreter dieser GPCR-Familie standen im Mittelpunkt dieser Arbeit: der Histamin H_2 Rezeptor (H_2R), der β_2-adrenerge Rezeptor (β_2AR) und der Histamin H_4 Rezeptor (H_4R). Die H_2R Antagonisten werden zur Therapie von peptischen Ulcera eingesetzt, die β_2AR Agonisten in der Behandlung des Asthma bronchiale. Der erste H_4R-Antagonist befindet sich gerade in der klinischen Prüfung zur Therapie des Asthma bronchiale und der allergischen Rhinitis. Weil alle drei GPCRs an vielen (patho)physiologischen Prozessen beteiligt sind, sind sie als Zielstrukturen für die Verbesserung bestehender Therapien und für die Etablierung neuer Therapieansätze interessant.

Ein erstes Ziel dieser Arbeit war die Analyse und der Vergleich H_2R- und β_2AR-induzierter Effekte an isolierten humanen neutrophilen Granulozyten. Aktivierung beider Rezeptoren in diesen Zellen führt zu einer Hemmung entzündlicher Prozesse, z. B. der Superoxid-Anionen ($O_2^{\bullet-}$) Bildung. Obwohl beide Rezeptoren klassisch an G_s Proteine koppeln, beeinflussten die aktivierten H_2R und β_2AR die Akkumulation von zyklischem Adenosin-3',5'-monophosphat (cAMP) und die $O_2^{\bullet-}$ Produktion in Neutrophilen sehr unterschiedlich. Ebenso wurden Unterschiede zu Daten aus rekombinanten Testsystemen gefunden. Ferner ergaben sich Hinweise auf Ligand-spezifische Rezeptorkonformationen, die für das Konzept der funktionellen Selektivität sprechen. Die Ergebnisse deuten darauf hin, dass die Beziehung zwischen der cAMP-Akkumulation und der Hemmung der $O_2^{\bullet-}$ Produktion in Neutrophilen komplexer ist als bisher angenommen. Das in dieser Arbeit beobachtete atypische Verhalten der H_2R-Antagonisten ist mit der klassischen Vorstellung der Interaktion zwischen einem GPCR und einem Antagonisten nicht vereinbar. Mögliche Erklärungen für die beobachteten H_2R- und β_2AR-induzierten Effekte werden diskutiert, wobei zusätzliche Untersuchungen notwendig sind, um die Unterschiede in den untersuchten Signalwegen in Neutrophilen mechanistisch zu verstehen.

Das zweite Ziel der Arbeit war die Charakterisierung monovalenter und bivalenter N^G-acylierter Hetarylpropylguanidine, hochpotenter (partieller) Agonisten am humanen H_2R in Sf9 Insektenzellen-Membranen, an humanen neutrophilen Granulozyten, da diese Zellen ein physiologisch relevanteres Testsystem darstellen. Diese H_2R Agonisten wurden zunächst als pharmakologische Werkzeuge entwickelt, könnten aber auch therapeutischen Nutzen haben, z. B. in der Therapie der akuten myeloischen Leukämie. In Gegensatz zu Histamin hemmten bivalente Acylguanidine die $O_2^{\bullet-}$ Produktion in den aktivierten Neutrophilen nicht. Ein monovalenter Ligand zeigte eine hemmende Wirkung auf die $O_2^{\bullet-}$ Produktion, die aber H_2R-unabhängig verlief. Dennoch stimulierten die untersuchten Liganden die cAMP Produktion über den H_2R, aber mit einer stark reduzierten agonistischen Wirkung im

Vergleich mit den im rekombinanten Testsystem bestimmten Wirkungen. Die ungünstigen physikalisch-chemischen Eigenschaften der N^G-acylierten Hetarylpropylguanidine (z. B. amphiphiler Charakter) müssten wahrscheinlich verbessert werden, um bessere Wirkungen am H_2R an Neutrophilen zu erzielen. Zusätzlich trägt diese Arbeit dazu bei, die Interaktionen der Acylguanidine mit dem H_2R auf molekularer Ebene besser zu verstehen. So zeigten die Untersuchungen des mutierten H_2R, dass der extrazelluläre N-Terminus bezüglich der unterschiedlichen Potenz und intrinsischen Aktivität der Acylguanidine am H_2R des Menschen und des Meerschweinchens nur eine marginale Rolle spielt.

Die präklinische Untersuchung neuer Wirkstoffe in translationalen Tiermodellen ist in der Arzneistoffforschung und -entwicklung unverzichtbar. Die Identifizierung und das Verständnis wesentlicher pharmakologischer Unterschiede zwischen H_4R Speziesorthologen ist daher essentiell. Im dritten Teil dieser Arbeit wurde die Bedeutung von extrazellulären Domänen für die unterschiedlichen pharmakologischen Eigenschaften des humanen (h) und des caninen (c) H_4R untersucht. Dafür wurden h/c-H_4R-Chimären generiert und in Sf9 Insektenzellen exprimiert. Unsere Daten deuten darauf hin, dass der N-Terminus und die erste extrazelluläre Schleife keinen Einfluss auf die Speziesunterschiede zwischen hH_4R und cH_4R haben. Dass die zweite und dritte extrazelluläre Schleife bei der H_4R-Aktivierung beteiligt sind, konnte erstmals gezeigt werden. Zum Beispiel ist die sehr niedrige Homologie der Aminosäuren im Bereich der letzten zwei extrazellulären Schleifen zumindest teilweise für die unterschiedlichen Effekt des Liganden JNJ7777120 (1-[(5-Chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazin) am hH_4R (inverser Agonist) und cH_4R (partieller Agonist) verantwortlich. Die vorgeschlagene Hypothese, dass die zweite und die dritte extrazelluläre Schleife in die inverse agonistische/agonistische Wirkung des JNJ7777120 involviert sind basiert auf molekulardynamischen Simulationen.

Insgesamt trägt diese Arbeit zur Erweiterung unserer Kenntnisse über die therapeutisch wichtigen H_2R, β_2AR und H_4R auf molekularer und/oder zellulärer Ebene bei.

Chapter 7

Supplementary Data

7.1 Sequencing results

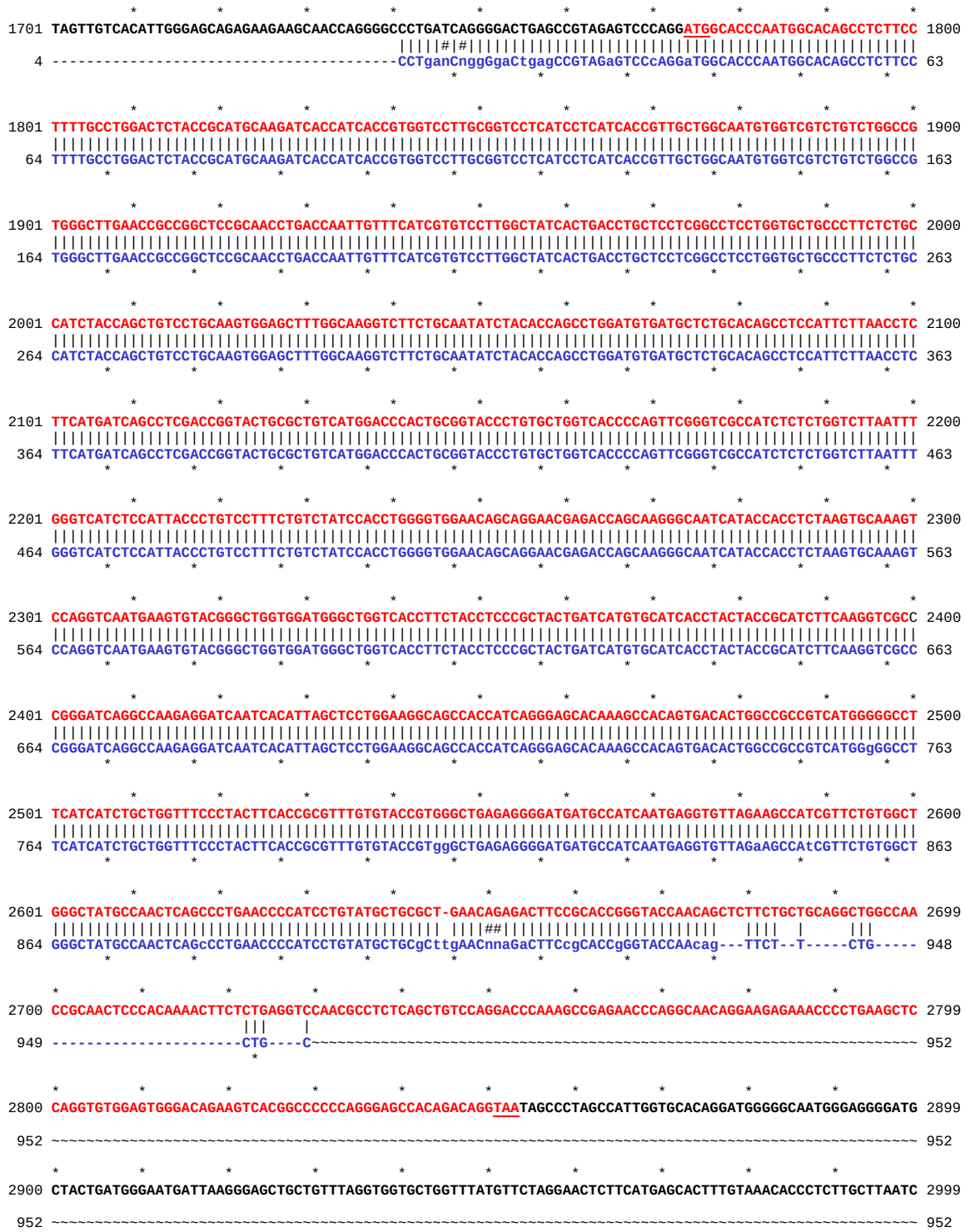

Fig. 7.1 Annealing of the nucleotide sequence of the hH$_2$R isoform 2 from NCBI database with the nucleotide sequence, obtained in sequencing using primer hH$_2$R_F_Iso2_seq. The encoding region of the hH$_2$R isoform 2 from NCBI database is shown in red (START and STOP codon are underlined) and part of the region before START and after STOP codon in black. The nucleotide sequence, obtained in sequencing, is shown in blue.

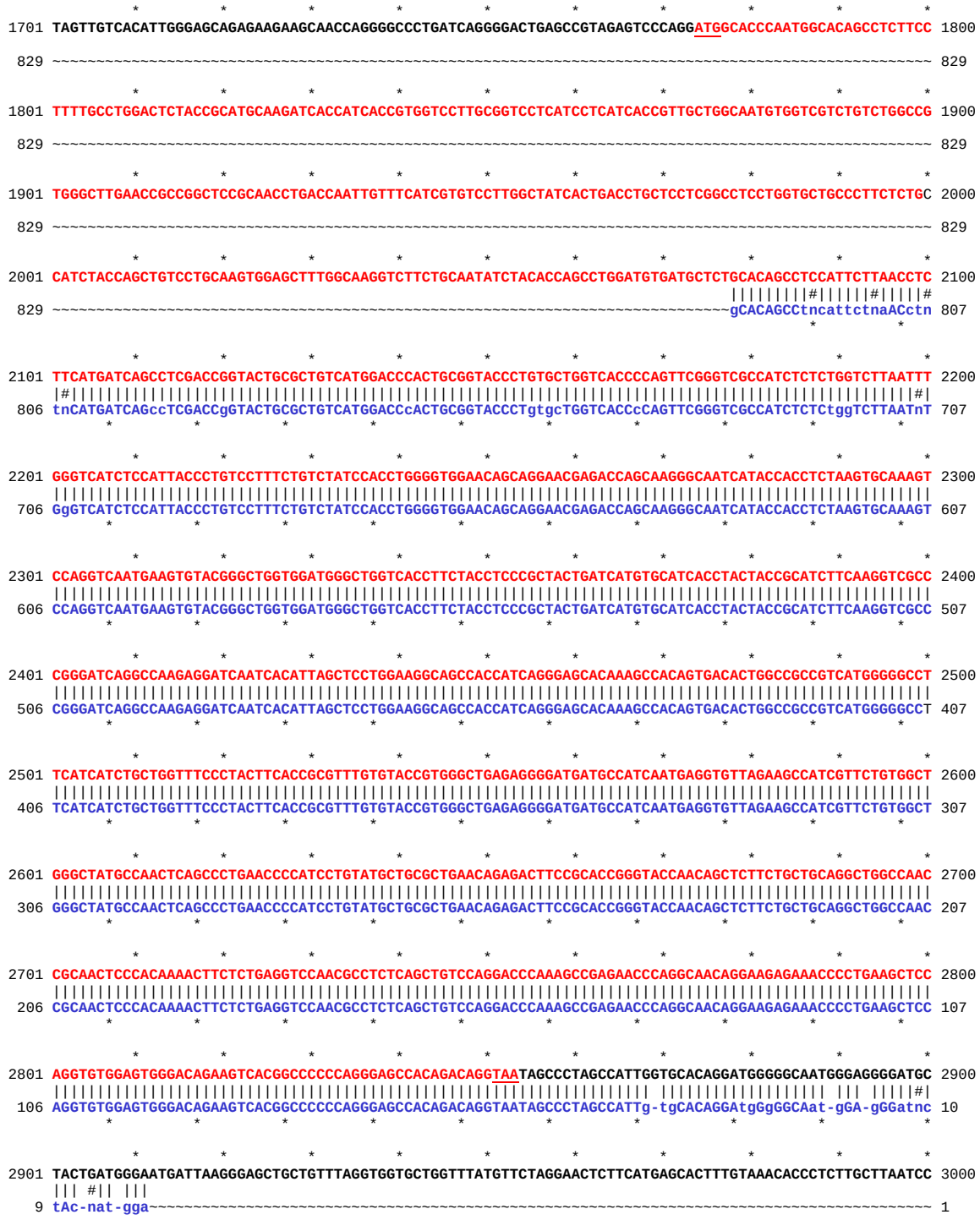

Fig. 7.2 Annealing of the nucleotide sequence of the hH$_2$R isoform 2 from NCBI database with the nucleotide sequence, obtained in sequencing using primer hH$_2$R_R_Iso2_seq. The encoding region of the hH$_2$R isoform 2 from NCBI database is shown in red (START and STOP codon are underlined) and part of the region before START and after STOP codon in black. The nucleotide sequence, obtained in sequencing, is shown in blue.

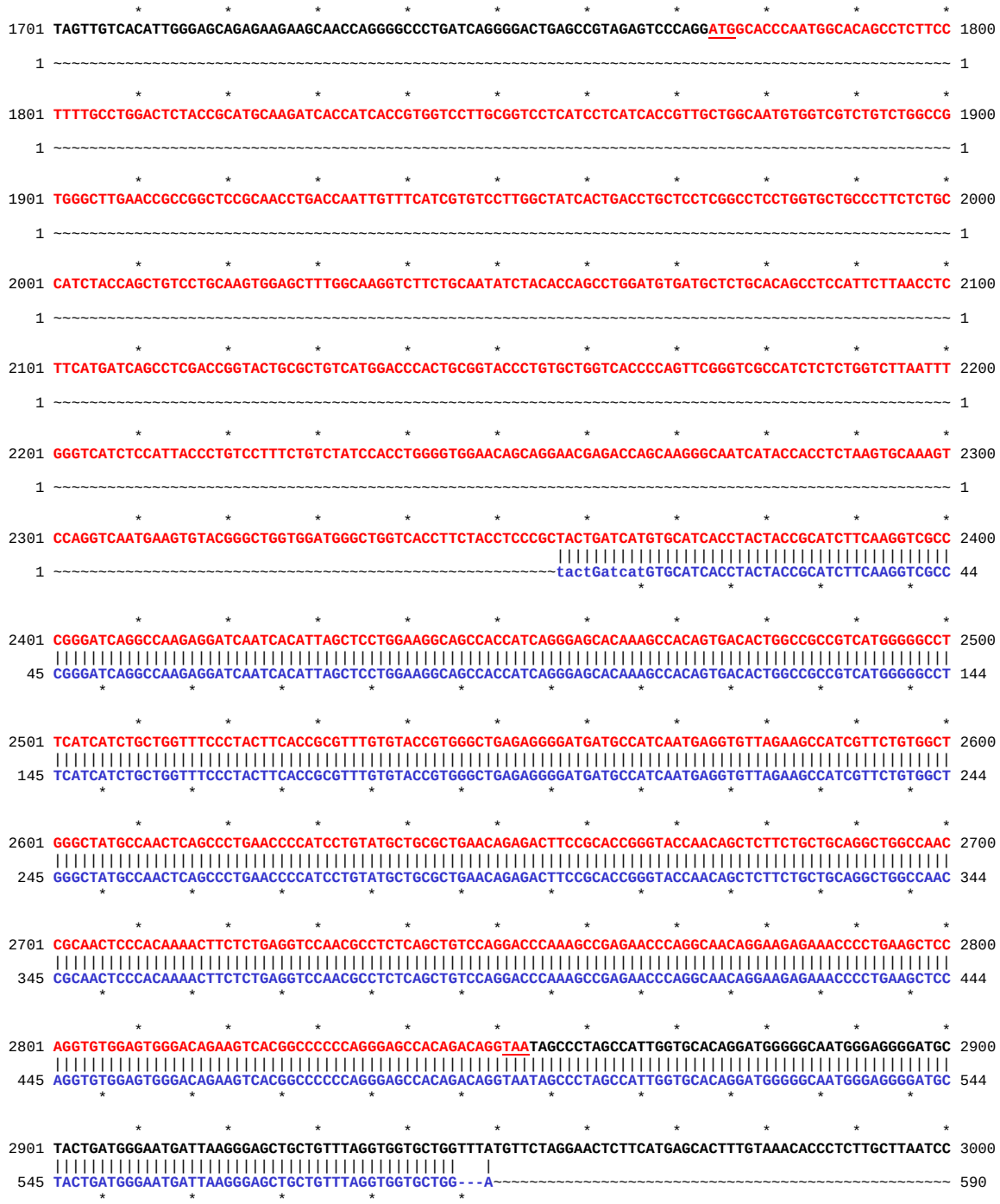

Fig. 7.3 Annealing of the nucleotide sequence of the hH_2R isoform 2 from NCBI database with the nucleotide sequence, obtained in sequencing using primer hH_2R_F_middle. The encoding region of the hH_2R isoform 2 from NCBI database is shown in red (START and STOP codon are underlined) and part of the region before START and after STOP codon in black. The nucleotide sequence, obtained in sequencing, is shown in blue.

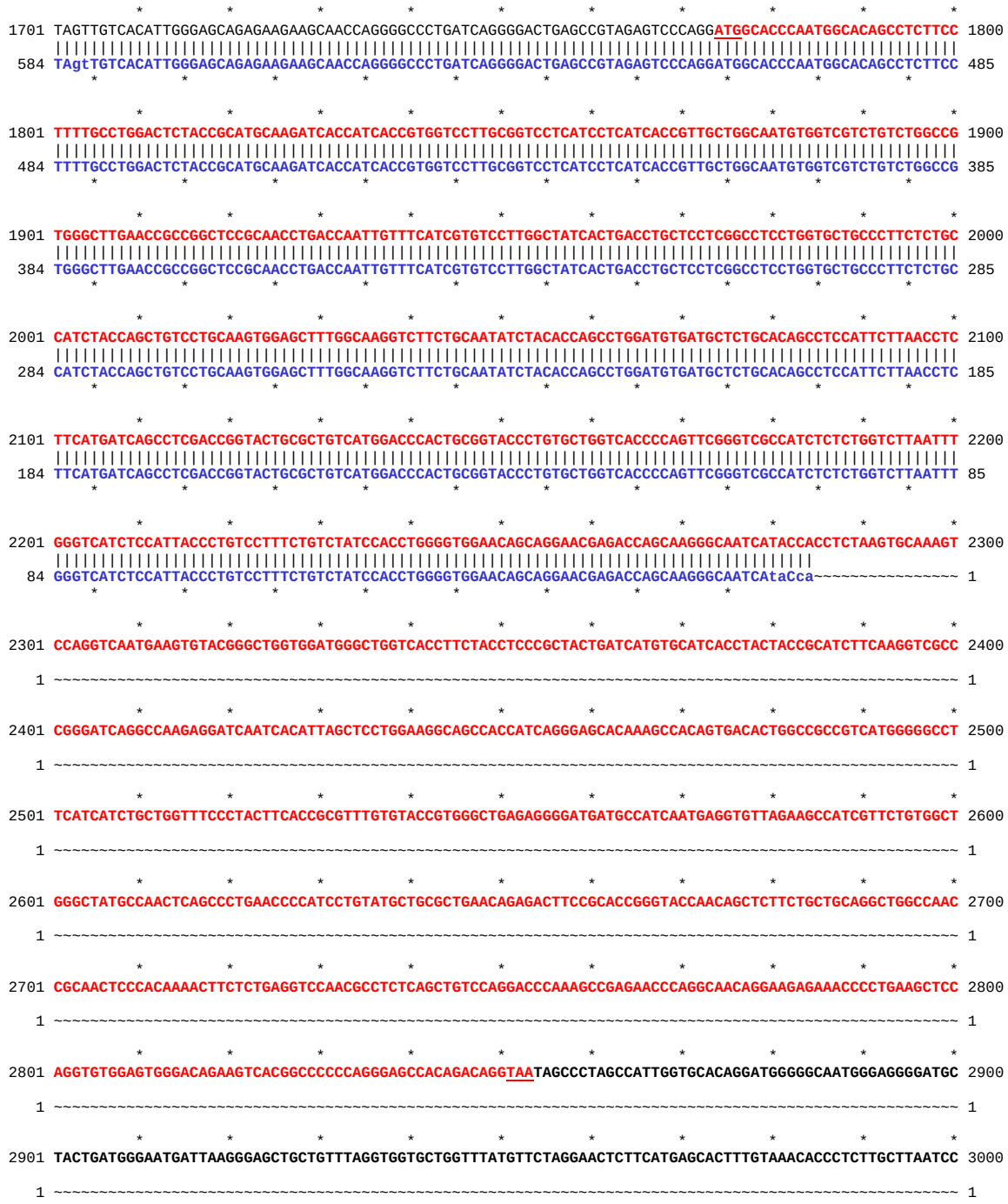

Fig. 7.4 Annealing of the nucleotide sequence of the hH$_2$R isoform 2 from NCBI database with the nucleotide sequence, obtained in sequencing using primer hH$_2$R_R_middle. The encoding region of the hH$_2$R isoform 2 from NCBI database is shown in red (START and STOP codon are underlined and part of the region before START and after STOP codon in black. The nucleotide sequence, obtained in sequencing, is shown in blue.

Chapter 8

Appendix

8.1 Publications, professional training and awards

8.1.1 Original publications (prior to submission of this thesis)

Birnkammer T, Spickenreither A, Brunskole I, Lopuch M, Bernhardt G, Dove S, Seifert R, Elz S and Buschauer A (**2011**) The bivalent ligand approach leads to highly potent and selective acylguanidine-type histamine H_2 receptor agonists. *J Med Chem* (in revision).

Brunskole I, Strasser A, Seifert R and Buschauer A (**2011**) Role of the second and third extracellular loops of the histamine H_4 receptor in receptor activation. *Naunyn Schmiedeberg's Arch Pharmacol* **384**:301-317.

Schnell D, Brunskole I, Ladova K, Schneider EH, Igel P, Dove S, Buschauer A and Seifert R (2011) Expression and functional properties of canine, rat, and murine histamine H_4 receptors in Sf9 insect cells. *Naunyn Schmiedeberg's Arch Pharmacol* **383**:457-470.

8.1.2 Reviews

Seifert R, Schneider EH, Dove S, Brunskole I, Neumann D, Strasser A and Buschauer A (2011) Paradoxical stimulatory effects of the "standard" histamine H_4 receptor antagonist JNJ7777120: the H_4 receptor joins the club of 7 transmembrane domain receptors exhibiting functional selectivity. *Mol Pharmacol* **79**:631-638.

8.1.3 Short lectures

Brunskole I, Straßer A, Seifert R and Buschauer A (**2011**) Second and third extracellular loop of histamine H_4 receptor are involved in receptor activation. DPhG Doktorandentagung, Heringsdorf, Germany and 50th annual meeting of the European Histamine Research Society (EHRS), Sochi, Russia. Abstract published in: *Inflamm Res* (2011).

Brunskole I, Straßer A, Seifert R and Buschauer A (**2011**) Histamine H_4 Receptor Species Differences: Role of Extracellular Domains. COST BM0806 MC/WG1-4 meeting, Krakow, Polen.

Brunskole I, Straßer A, Buschauer A and Seifert R (**2010**) Extracellular loops 2 and 3 do contribute to species differences between human and canine histamine H_4 receptor. 5th Summer School Medicinal Chemistry, Regensburg, Germany.

8.1.4 Poster presentations

Brunskole I, Straßer A, Buschauer A and Seifert R (**2010**) Extracellular loops 2 and 3 do contribute to species differences between human and canine histamine H_4 receptor. 5th Summer School Medicinal Chemistry, Regensburg, Germany.

Brunskole I, Straßer A, Buschauer A and Seifert R (**2010**) Contribution of extracellular domains to ligand-receptor interactions at the human histamine H_4 receptor. 51. Jahrestagung der Deutschen Gesellschaft für Experimentelle und Klinische Pharmakologie und Toxikologie (DGPT), Mainz, Germany.

Brunskole I, Straßer A, Seifert R and Buschauer A (**2009**) Role of Extracellular Domains of the Histamine H_4 Receptor in Receptor Activation. Jahrestagung der Deutschen Pharmazeutischen Gesellschaft (DPhG), Jena, Germany.

8.1.5 Professional training

10/2008 – 09/2011	Member of the Research Training Group (Graduiertenkolleg 760) "*Medicinal Chemistry: Molecular Recognition – Ligand Receptor Interactions*" of the German Research Foundation. *Regensburg, Germany*
03/2010	Advanced Courses in Pharmacology, Teil I: Methoden zur Analyse G Protein-gekoppelter Rezeptoren, Teil II: Moderne Massenspektrometrie in Pharmakologie und Toxikologie. *Mainz, Germany*
03/2009 – 10/2009	Weiterbildung „Versuchstierkunde und Tierschutz" (Bestandteil des Nachweises der Sachkunde für den Umgang mit Versuchstieren innerhalb der EU, FELASA Kategorie B). *Regensburg, Germany*
03/2009	Fortbildung für Projektleiter und Beauftragte für Biologische Sicherheit (§15 und 17 Gentechniksicherheitsverordnung). *Regensburg, Germany*
01/2009	Umgang mit offenen radioaktiven Stoffen. *Regensburg, Germany*

8.1.6 Awards

Brunskole I, Straßer A, Buschauer A and Seifert R (**2010**) Extracellular loops 2 and 3 do contribute to species differences between human and canine histamine H_4 receptor. **Poster Award** in occasion of the 5th Summer School Medicinal Chemistry, Regensburg, Germany.

8.2 Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet. Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Regensburg, den _____ _____

Irena Brunskole