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PERSPECTIVE

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

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ABSTRACT

The histamine H_4 receptor (H_4R) is expressed in several cell types of the immune system and is assumed to play an important pro-inflammatory role in various diseases, including bronchial asthma, atopic dermatitis, and pruritus. Accordingly, H_4R antagonists have been suggested to provide valuable drugs for the treatment of these diseases. Over the past decade, the indole derivative 1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine (JNJ7777120) has become the "standard" H_4R antagonist and has been extensively used to assess the pathophysiological role of the H_4R. However, the situation has now become more complicated by recent data (p. 749 and *Naunyn Schmiedebergs Arch Pharmacol* doi: 10.1007/s00210-011-0612-3) showing that JNJ7777120 can also activate β-arrestin in a supposedly G_i-protein-independent (pertussis toxin-insensitive) manner and that at certain H_4R species orthologs, JNJ7777120 exhibits partial agonist efficacy with respect to G_i-protein activation (steady-state high-affinity GTPase activity). These novel findings can be explained within the concept of functional selectivity or biased signaling, assuming unique ligand-specific receptor conformations with distinct signal transduction capabilities. Thus, great caution must be exerted when interpreting in vivo effects of JNJ7777120 as H_4R antagonism. We discuss future directions to get out of the current dilemma in which there is no "standard" H_4R antagonist available to the scientific community.

Introduction

Histamine (Fig. 1) is an important neurotransmitter and local mediator (Hill et al., 1997). A decade ago, several groups each independently identified a novel member of the H_xR family with unique pharmacological properties, the H_4R (for review, see Hough, 2001; Thurmond et al., 2008; Leurs et al., 2009). The H_4R is a G_i-protein-coupled receptor, causing inhibition of adenylyl cyclase and, in cells of the immune system, activation of phospholipase C via release of $G\beta\gamma$-complexes (Fig. 2A). In cell membranes, activation of G_i-proteins by the H_4R can be monitored by histamine-stimulated [^{35}S]GTPγS binding to, or [γ-^{32}P]GTP hydrolysis by, G_i-proteins (Schneider et al., 2009). The discovery of the H_4R was highlighted in a *Perspective* article in *Molecular Pharmacology* (Hough, 2001). In this very first review-type pub-

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ABBREVIATIONS: GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; JNJ7777120, 1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine; 7TM, seven-transmembrane domain; H_xR, histamine H_1- H_2-, H_3 or H_4 receptor; c, canine; h, human; m, mouse; r, rat; PTX, pertussis toxin; ERK, extracellular signal-regulated kinase; UR-PI376, 2-cyano-1-[4-(1*H*-imidazol-4-yl)butyl]-3-[(2-phenylthio)ethyl]guanidine; UR-PI294, N^1-[3-(1*H*-imidazol-4-yl)propyl]-N^2-propionylguanidine.

lication on this topic, it was already noted that the pharmacological properties of the H_4R, although clearly distinct from those of other H_xRs, differed from each other in various studies. Most notably, one group reported on relatively high affinity of H_4R for H_1R antagonists but other groups found no interaction of the H_4R with H_1R antagonists (Hough, 2001; Nguyen et al., 2001). Despite considerable efforts, these early discrepancies have not been satisfactorily explained (Deml et al., 2009). Now, again, the H_4R causes headache concerning its pharmacological properties.

The H_4R is expressed in several cell types of the immune system, including mast cells, eosinophils, dendritic cells, and T lymphocytes. On the basis of this localization of the receptor and studies with the H_4R knockout mouse, it has been suggested that the H_4R plays a proinflammatory role in bronchial asthma, atopic dermatitis, and pruritus and that H_4R antagonists could be useful drugs for the treatment for these conditions (see, e.g., Thurmond et al., 2008; Leurs et al., 2009). This suggestion has been corroborated by the finding that the indole derivative 1-[(5-chloro-1*H*-indol-2-yl) carbonyl]-4-methylpiperazine (JNJ7777120) (Fig. 1), a potent H_4R antagonist (Jablonowski et al., 2003; Venable et al., 2005), exhibits anti-inflammatory effects in a mouse asthma model (Dunford et al., 2006). Moreover, JNJ7777120 inhibits the effects of histamine in various cell systems expressing the H_4R (see, e.g., Thurmond et al., 2008; Leurs et al., 2009).

Although JNJ7777120 has a relatively short plasma half-life and limited bioavailability, rendering animal experiments requiring continuous exposure to the compound technically difficult (Thurmond et al., 2004), researchers in the H_4R field readily embraced JNJ7777120 because of its availability and high H_4R selectivity (Jablonowski et al., 2003; Venable et al., 2005). Table 1 summarizes pharmacological data for JNJ7777120 in various in vitro test systems. The implementation of JNJ7777120 as "standard" H_4R antagonist was also facilitated by the fact that the first known standard H_4R antagonist, thioperamide (Fig. 1), is not selective for the H_4R but is also a potent H_3R antagonist, rendering it potentially difficult to discriminate H_3R and H_4R effects in vivo. More precisely, thioperamide is a dual H_3R/H_4R inverse agonist; i.e., according to the two-state model of 7TM receptor activation, the compound stabilizes the inactive R state, resulting in a reduction of basal G-protein activation promoted by the agonist-free H_3R and H_4R (Schneider et al., 2009; Schnell et al., 2010). Numerous H_4R ligands structurally related to JNJ7777120 have been synthesized (Venable et al., 2005); with few exceptions (Schneider et al., 2010), these compounds have not yet been characterized in depth pharmacologically.

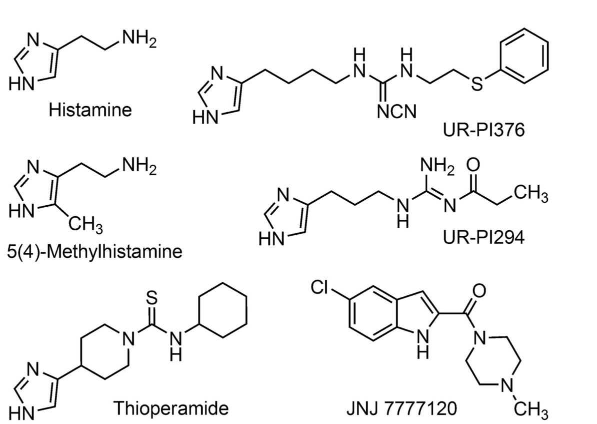

Fig. 1. Structures of selected H_4R ligands analyzed in the present study. According to conventional definition, histamine, 5(4)-methylhistamine, UR-PI294, and UR-PI376 are H_4R agonists, whereas thioperamide and JNJ7777120 are H_4R antagonists. The classification of the compounds depends substantially on the specific system analyzed, and most strikingly, even the "standard" antagonists JNJ7777120 and thioperamide can exhibit agonistic effects at H_4R, reflecting functional selectivity or biased signaling.

Activation of β-Arrestin by JNJ7777120: A Twisted Story

On this basis, Rosethorne and Charlton (2011), in this issue of *Molecular Pharmacology*, provide important insights into the pharmacological properties of the hH_4R in general and into the properties of JNJ7777120 in particular. Using the U2OS osteosarcoma cell expression system, the authors show that, as expected (Schneider et al., 2009), the endogenous and full agonist histamine stimulates $[^{35}S]GTP\gamma S$ binding in cell membranes, whereas the inverse agonist thi-

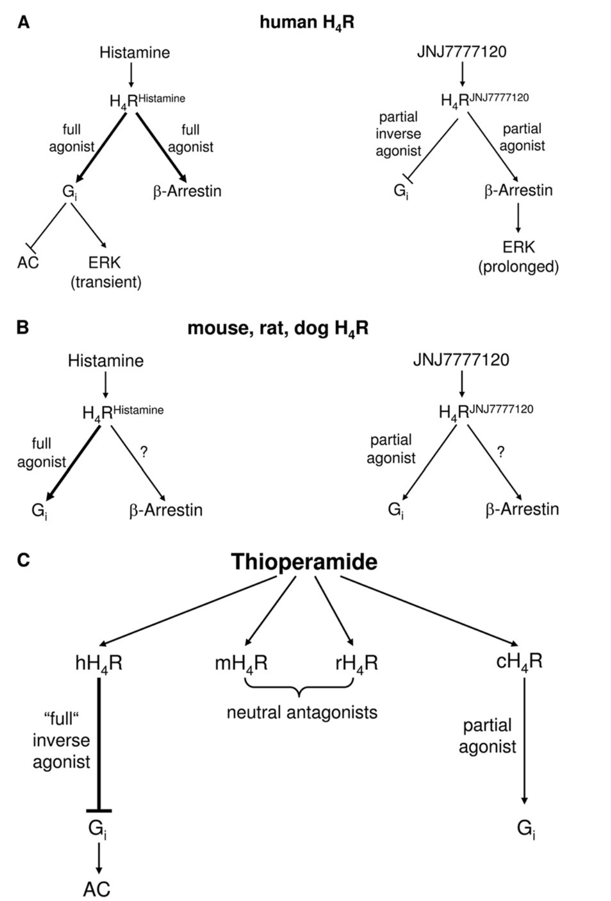

Fig. 2. Functional selectivity of JNJ7777120 and thioperamide. A, at hH_4R, histamine is a full agonist with respect to G_i- and β-arrestin activation. JNJ7777120 is a partial inverse agonist with respect to G_i-protein activation and a partial agonist with respect to β-arrestin activation. B, at mH_4R, rH_4R, and cH_4R, Histamine is a full agonist with respect to G_i activation in Sf9 insect cell membranes, whereas JNJ7777120 is a partial agonist at these H_4R orthologs. The effects on β-arrestin activation have not yet been studied. C, considering the effects of thioperamide on G_i-protein activation catalyzed by various H_4R species orthologs, effects may range from "full" inverse agonism [even thioperamide is actually not a full inverse hH_4R agonist (Schneider et al., 2009)] over neutral antagonism to partial agonism.

operamide effectively reduces [^{35}S]GTPγS binding, reflecting constitutive activity of hH_4R. With respect to [^{35}S]GTPγS binding, JNJ 777120 exhibits, to a variable degree, partial inverse agonist or neutral antagonist properties at hH_4R (Schneider et al., 2009, 2010; Rosethorne and Charlton, 2011) (Fig. 2A; Table 1). In addition to stimulation of [^{35}S]GTPγS binding, histamine also stimulates binding of β-arrestin to hH_4R. β-Arrestin recruitment to 7TM receptors has traditionally been linked to receptor uncoupling from G-proteins and desensitization (Luttrell and Gesty-Palmer, 2010; Rajagopal et al., 2010). More recently, however, it has become clear that β-arrestin can also serve as signal-transducing protein, stimulating G-protein-independent signal transduction pathways such as ERK (Luttrell and Gesty-Palmer,

TABLE 1

Characterization of JNJ7777120 in different in vitro test systems

JNJ7777120 is the best-studied selective H_4R ligand. The table summarizes the data of important studies characterizing the compound in in vitro test systems. Information on the expression system, the parameter (functional assay or radioligand binding)) measured and pharmacological parameters is provided. α Designates the efficacy of the ligand. To facilitate comparison of the data from various studies, K_i, IC_{50} and EC_{50} values are all provided in molar units. For functional inhibition experiments, the concentration of the stimulus histamine is provided as well.

Expression System	Assays	Important Findings Concerning JNJ7777120	Reference
Human SK-N-MC cells	Radioligand binding cAMP-CRE gene reporter assay	Human H_4R, [^{3}H]histamine competition binding: K_i = 4.1 nM, K_d = 4 nM and pA_2 = 7.9 nM cAMP assay with human, mouse, rat H_4R: equipotent antagonistic potency; 1000-fold selectivity relative to H_1R, H_2R, and H_3R no cross-reactivity with 50 other targets	Jablonowski et al., 2003
Mouse bone marrow-derived mast cells	In vitro mast cell chemotaxis assay	Antagonist: inhibition of chemotaxis in mast cells, induced by 10 μM of histamine: IC_{50} = 40 nM	Thurmond et al., 2004
Endogenous (human eosinophils)	Flow cytometry, in vitro chemotaxis assays	Antagonist: inhibition of eosinophil shape change, induced by 1 μM of histamine: IC_{50} = 300 nM; inhibition of chemotaxis, induced by 1 μM of histamine: IC_{50} = 86 nM	Ling et al., 2004
	Fluorescence imaging	Antagonist: inhibition of actin polymerization, induced by 300 nM of histamine: IC_{50} = 6 nM	Barnard et al., 2008
Sf9 insect cells	Radioligand binding, steady-state GTPase assay	Partial inverse agonist at human H_4R: EC_{50} = 37.7 ± 8.5 nM; α = −0.31 (related to the efficacy of thioperamide)	Schneider et al., 2009
		Partial agonist at mouse (EC_{50} = 186 nM; α = 0.61), canine (EC_{50} = 155 nM; α = 0.66) and rat H_4R (EC_{50} = 316 nM; α = 0.51) in the GTPase assay (G_i-protein activation)	Schnell et al., 2011
		Differences between K_i value in [^{3}H]histamine competition binding (18.6 nM) and EC_{50} value with respect to inverse agonistic activity in the GTPase assay (77.6 nM); for other compounds structurally related to JNJ7777120, even larger differences were found	Schneider et al., 2010
Human HEK 293/ HEK 293T cells	Radioligand binding ([^{3}H]histamine competition)	Substantial affinity differences of JNJ7777120 at various species isoforms: human (K_i = 5 nM), monkey (K_i = 32 nM), pig (K_i = 501 nM), dog (K_i = 79 nM), mouse (K_i = 4 nM), rat (K_i = 4 nM) and guinea pig H_4R (K_i = 1 μM)	Lim et al., 2010
	SRE-luciferase reporter gene assay ($cH_4R + G\alpha_{qi}$)	Antagonist at canine H_4R: rightward shift of the histamine concentration-response-curve, no pA_2 reported	Jiang et al., 2008
COS-7 cells	Radioligand binding	[^{3}H]histamine competition binding assay: K_i: 50 nM	
Human U2OS cells	GTPγS binding assay, β-arrestin recruitment assay, ERK phosphorylation	Weak partial inverse agonist in [^{35}S]GTPγS binding assay (EC_{50} = 79 nM and α = −0.05, TABLE 2). However, in Fig. 1, JNJ7777120 appears to be a neutral antagonist with respect to [^{35}S]GTPγS binding; partial agonist: increase in recruitment of β-arrestin in a supposedly G-protein independent (PTX-insensitive) manner (EC_{50} = 12.5 nM and α = 0.64); effective and prolonged ERK activation at a very high ligand concentration (100 μM!)	Rosethorne and Charlton, 2011
Mouse pituitary tumor AtT-20 cells	Adrenocorticotropin release (ELISA)	Antagonist: inhibition of ACTH secretion, induced by histamine (10 nM) or R-α-methylhistamine (100 nM): IC_{50} = 360 or 230 nM, respectively	Meng et al., 2008

HEK, human embryonic kidney; ELISA, enzyme-linked immunosorbent assay.

2010; Rajagopal et al., 2010). For this reason, GPCRs should actually be more correctly referred to as 7TM receptors, giving credit to the universal heptahelical structure of these proteins instead of their signal transduction pathways, which are not necessarily G-protein-mediated.

So far, the data of Rosethorne and Charlton (2011) fit into established paradigms, but the headache starts with their finding that JNJ7777120 behaves as a partial agonist with respect to β-arrestin binding to hH_4R (Fig. 2A; Table 1). This effect was observed at various H_4R expression levels, ruling out the possibility that excess H_4R molecules, referred to as receptor reserve, could account for the unexpected effects. Moreover, the effect of JNJ7777120 seems to be G_i-protein-independent, as suggested by the lack of influence of the ADP-ribosyltransferase PTX on β-arrestin binding. The authors provide evidence that the effect of JNJ7777120 on β-arrestin recruitment is mediated by the H_4R and not through another receptor. Specifically, thioperamide, which is without stimulatory effect in this assay by itself, blocks the effects of JNJ7777120 on β-arrestin recruitment competitively, and the pA_2 values of thioperamide for blockade of the JNJ7777120 response and the response of the H_4R agonist clobenpropit are very similar. Unfortunately, the pA_2 for the endogenous H_4R ligand histamine was not reported. This is not trivial because the apparent affinity of thioperamide may be ligand-dependent.

Most striking is the finding that β-arrestin binding to hH_4R is not a dead-end. In particular, JNJ7777120 induces very effective and prolonged ERK activation, whereas histamine induces only transient ERK activation. These time courses are typical for arrestin- and G-protein-dependent signal transduction, respectively (Luttrell and Gesty-Palmer, 2010), but the two pathways were not dissected with PTX by Rosethorne and Charlton (2011). In any case, these data show that JNJ7777120 is capable of stabilizing a conformation in hH_4R that induces β-arrestin recruitment and stimulates an important downstream signaling pathway, at least in an osteosarcoma cell line. Thus, JNJ7777120 cannot be considered the "standard" H_4R antagonist anymore, but depending on the parameter assessed, JNJ7777120 may also act as agonist. It is noteworthy that, with respect to β-arrestin recruitment, JNJ7777120 acts only as partial agonist, but with respect to ERK activation, JNJ7777120 is actually a full agonist. However, the stimulatory effects of JNJ7777120 on ERK activation was only reported for an exceedingly high ligand concentration (100 μM), a concentration that is almost 10,000-fold higher than the EC_{50} for β-arrestin recruitment (Table 1). Thus, one cannot exclude the possibility that in addition to β-arrestin, other signal transduction pathways are involved in JNJ7777120-induced ERK activation. It will now be very important to study in great detail the effect of JNJ7777120 on ERK activation in more commonly used and well characterized expression systems such as HEK293 cells and in cells endogenously expressing hH_4R. Eosinophils are a well established native cell system for studying hH_4R functions (Table 1). It is likely that the effects of JNJ7777120 strongly depend on the endogenous complement of signal transduction proteins in the cells harboring hH_4R.

Additional evidence that JNJ7777120 and structurally related compounds are more than just H_4R "antagonists" comes from a recent analysis of a series of 25 indole, benzimidazole, and thienopyrrole compounds at the recombinant hH_4R expressed in Sf9 insect ells (Schneider et al., 2010). In this system, for a subset of compounds including JNJ7777120, we observed quite substantial differences between K_i values in [^{3}H]histamine competition binding studies and EC_{50} values for inverse agonistic activity in the GTPase assays (Schneider et al., 2010) (Table 1). Although the data on JNJ7777120 compiled in Table 1 have to be compared with caution because they were obtained in different cell types and because different parameters were determined, it is evident that the apparent affinities/potencies of JNJ7777120 can vary considerably among the various studies, a property that is not commonly observed for classic receptor antagonists. For example, in the studies of Ling et al. (2004) and Barnard et al. (2008), the IC_{50} values for JNJ7777120 on various functional parameters in human eosinophils endogenously expressing hH_4R differ by up to 50-fold (Table 1). These differences cannot be explained by the relatively small differences in the stimulatory histamine concentration used. Such data on divergent ligand affinities/potencies regarding various parameters support the notion that JNJ7777120 and related compounds stabilize functionally distinct hH_4R conformations.

Although the data from Rosethorne and Charlton (2011) are certainly unexpected for the H_4R community and raise many questions of how JNJ7777120 effects in vitro and particularly in vivo should be interpreted, in a broader conceptual context, the findings are actually not that surprising. It is just that the H_4R now joins the growing family of 7TM receptors showing functional selectivity or biased signaling. This concept states that any given ligand stabilizes a unique conformation in a particular 7TM receptor that is then capable of activating a unique pattern of G-protein-dependent and -independent signal transduction pathways (Galandrin et al., 2007; Rajagopal et al., 2010). β-Adrenergic receptor antagonists are a very prominent and well studied class of ligands for which functional selectivity has been documented (Galandrin et al., 2007).

Even More Twists with JNJ7777120, Extending to Thioperamide

But the JNJ7777120 story has some additional unexpected twists. First, we have recently studied the effects of JNJ7777120 at recombinant hH_4R, mH_4R, and cH_4R expressed in Sf9 cells, using the steady-state high-affinity GTPase assay as parameter (Schnell et al., 2011). It is important to characterize the effects of JNJ7777120 at those H_4R species orthologs because mouse, rat, and dog are important laboratory animal species for assessing the pathophysiological role of the H_4R (Liu et al., 2001; Dunford et al., 2006). Most strikingly, at mH_4R, rH_4R, and cH_4R expressed in S9 cells, JNJ7777120 exhibits strong partial agonism with respect to activation of G_i-proteins (Fig. 2B). However, a comparison of the efficacies must consider the constitutive activity, which is very high in the case of the hH_4R and very low for the mH_4R, rH_4R and cH_4R (Schnell et al., 2011). Thus, JNJ7777120 may lead to a similar equilibrium of active and inactive states for all H_4R orthologs, which appears as inverse agonism at hH_4R because of the shift of the basal equilibrium toward the active state (R*), and as partial agonism at mH_4R, rH_4R, and cH_4R because of the different basal equilibria (presence or absense, respectively, of the R* state). The effect of JNJ7777120 on β-arrestin activation has not yet been studied with these H_4R species orthologs.

And then there is still another twist extending to thioperamide. To this end, in all studies including the study of Rosethorne and Charlton (2011), there has been consensus that thioperamide acts as H_4R inverse agonist (Leurs et al., 2009; Schneider et al., 2009). However, at cH_4R, thioperamide clearly exhibits partial agonistic efficacy, and at mH_4R and rH_4R, thioperamide is a neutral antagonist with respect to G_i-protein activation (Schnell et al., 2011). Thus, depending on the species studied, thioperamide can stabilize either only inactive or both inactive and active H_4R conformations, and the functional behavior of this compound is clearly different from the behavior of JNJ7777120 (Fig. 2, B and C). It is also possible that the controversial effects of H_1R antagonists observed at the H_4R (Hough, 2001; Nguyen et al., 2001; Deml et al., 2009) are due to functional selectivity. At least, the H_1R antagonist data are reminiscent of what has been observed for JNJ7777120 (Hough, 2001; Deml et al., 2009) (Table 1), and a careful analysis of this problem is warranted.

What Is the Molecular Basis for the Divergent Effects of JNJ7777120 on Various Signaling Pathways Promoted by hH_4R? Also a Twisted Story

We have developed a model of the interaction of JNJ7777120 and related compounds with hH_4R (Schneider et al., 2010). We suggested that JNJ7777120 prevents the indole ring of $Trp^{6.48}$, the key element of the proposed 7TM receptor-activating toggle switch, from changing the inactive vertical position into the horizontal position (Schneider et al., 2010). However, JNJ7777120 stabilizes both inactive and active hH_4R states (Fig. 2). Therefore, the compound was docked into models of the inactive hH_4R (Fig. 3A) and of the active hH_4R (Fig. 3B). The positively charged amine moiety of JNJ7777120 interacts electrostatically with the highly conserved $Asp^{3.32}$ in both states. In the inactive hH_4R, the indole moiety of JNJ7777120 adopts a nearly vertical position with respect to the longitudinal axis of the receptor, placed between transmembrane domains III and VI. This conformation stabilizes the indole ring of $Trp^{6.48}$ in a vertical position, too (Fig. 3A). These assumptions correspond to published data of related compounds (Schneider et al., 2010). For the active state of the hH_4R, the docking studies suggest an alternative binding mode of JNJ7777120 in which the indole moiety is stacked between the aromatic side chains of $Trp^{6.48}$ and $Tyr^{6.51}$. This interaction stabilizes the indole ring of $Trp^{6.48}$, discussed as being involved in the rotamer toggle switch during receptor activation, in a more horizontal position (Fig. 3B). Based on the present models, no differences in amino acids directly interacting with JNJ7777120 in the binding pocket of inactive and active hH_4R are obvious. Unfortunately, no conclusions can be drawn from the models about the molecular mechanism by which JNJ7777120 induces and stabilizes a β-arrestin-binding conformation of the hH_4R, but in the following, we will propose a mechanism based on the available literature.

Nonvisual arrestins 2 and 3 preferentially bind to active phosphorylated 7TM receptors but, e.g., in the case of β_2 adrenergic and M_2 muscarinic receptors, also to phosphorylated inactive states (~2-fold lower binding; Gurevich and Gurevich, 2006). However, unphosphorylated receptor states must be active to bind arrestins with sufficient affinity. Thus, recruitment of β-arrestin to an inactive (not G-protein coupled) receptor state requires phosphorylation of multiple serine and threonine residues in the intracellular loops and the C-terminal tail, 7TM receptor regions that all have been shown to contain phosphorylation sites relevant for arrestin binding (Gurevich and Gurevich, 2006). 7TM receptors are mainly phosphorylated by GRKs. Among them, GRKs 5 and 6 are independent of translocation by Gβγ-subunits and exclusively responsible for ERK1/2 activation by arrestins (Kim et al., 2005; Ren et al., 2005).

Because the JNJ7777120-mediated recruitment of β-arrestin supposedly does not depend on G-proteins (Rosethorne and Charlton, 2011), JNJ7777120 may stabilize a specific hH_4R state that does not activate G_i-proteins but in the first place facilitates phosphorylation of newly exposed serine and threonine residues by Gβγ-independent GRKs. Some of the phosphorylated serine/threonine sites or clusters may then interact with the lysine- and arginine-rich polar core of β-arrestin. Specific phosphorylation patterns differentially orient β-arrestin on the receptor and stabilize different active arrestin conformations, leading to structurally and functionally distinct arrestin-receptor complexes (Gurevich and Gurevich, 2006). To enable high-affinity hH_4R-binding and activation of such a functionally distinct β-arrestin state resulting in prolonged ERK activation (Rosethorne and Charlton, 2011), JNJ7777120 should therefore expose the proper pattern of hH_4R phosphorylation sites by conformational changes in the intracellular loops and the C-terminal tail. These conformational changes may also enable interactions with additional

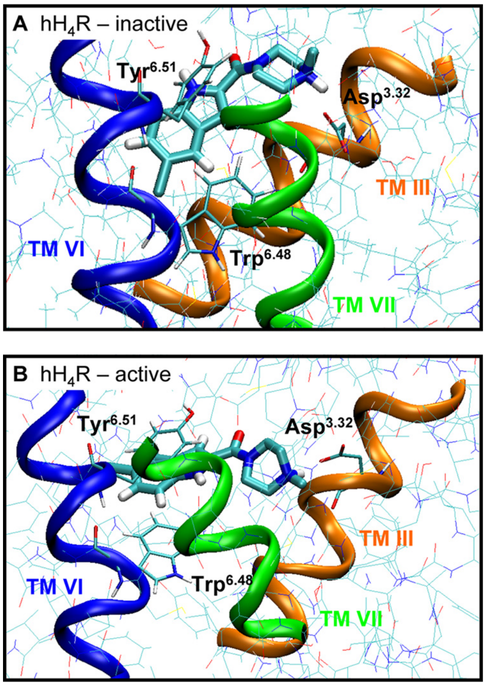

Fig. 3. Model of the interaction of JNJ7777120 with inactive and active hH_4R. Homology models of the inactive and active hH_4R were generated as described previously (Schneider et al., 2010). For modeling of the inactive hH_4R, the crystal structure of the β_2 adrenergic receptor (Protein Data Bank 2RH1) was used as template, whereas for modeling of the active hH_4R, the crystal structure of active opsin (Protein Data Bank 3DQB) was applied. JNJ7777120 was docked into both models, using SYBYL 7.3 (Tripos, St. Louis, MO). A, JNJ7777120 docked into the inactive hH_4R. B, JNJ7777120 docked into the active hH_4R.

arrestin regions, contributing to the activation of the complex via induced fit.

And Even More Twists: Are the Effects of JNJ7777120 on β-Arrestin Recruitment Really G-Protein-Independent?

The conclusion that JNJ7777120 induces β-arrestin recruitment in a G-protein-independent manner is based on the finding that the ligand does not stimulate [^{35}S]GTPγS binding and on the lack of effect of PTX on β-arrestin recruitment (Rosethorne and Charlton, 2011). PTX, via ADP-ribosylation of G_i-protein α-subunits, uncouples 7TM receptors from G_i-proteins and is a most valuable tool for unmasking G_i-protein-dependent pathways (Ui and Katada, 1990). Although inhibitory effects of PTX on receptor-mediated signaling can be readily interpreted [specifically whether the proper controls with the B-oligomer (and not only the carrier solvent) are performed], the interpretation of negative PTX data is more complicated. In particular, it is difficult to completely ADP-ribosylate all G_i-protein α-subunits in cells because G_i-proteins are so abundant (Ui and Katada, 1990). For this reason, PTX effects are often only incomplete. Accordingly, one cannot exclude the possibility that a fraction of the available G_i-proteins in the osteosarcoma cell expression system used by Rosethorne and Charlton (2011) was still functional and, evidently, those non–ADP-ribosylated G_i-proteins could participate in signaling, including β-arrestin recruitment and ERK activation (Walters et al., 2009). At the membrane level, activation of G_i-protein may be too small to be detected, but the β-arrestin assay may be sufficiently sensitive to detect G_i-protein activation.

Rosethorne and Charlton (2011) incubated the cells for 20 h with 200 ng/ml PTX, but even a 24-h incubation with PTX at a concentration of 1 μg/ml may be insufficient to functionally eliminate all G_i-proteins from receptor-coupling (Ui and Katada, 1990). It is possible to assess the effectiveness of PTX-catalyzed ADP-ribosylation by treating membranes from PTX-treated cells with preactivated PTX and [^{32}P]NAD and then performing SDS polyacrylamide electrophoresis with subsequent quantitative autoradiography. Effective ADP-ribosylation of G_i-proteins in intact cells results in poor incorporation of [^{32}P]ADP-ribose in the subsequent membrane incubation with activated PTX. Unfortunately, this important control experiment was not performed in the study of Rosethorne and Charlton (2011).

Moreover, the specific activity of PTX from various commercial suppliers can be quite different and vary considerably from batch to batch, so that it is always essential to include a positive control experiment to document functionality of PTX. Unfortunately, the source of PTX is not mentioned in the study by Rosethorne and Charlton (2011), and the authors also did not present a positive control experiment showing that PTX actually functioned properly. Such a positive control could have been provided by demonstrating a lack of stimulatory effect of histamine on [^{35}S]GTPγS binding in membranes from PTX-treated cells and a decrease in basal [^{35}S]GTPγS binding, reflecting uncoupling of constitutively active receptors from G_i-proteins (Seifert and Wenzel-Seifert, 2003).

Considering the constitutive activity of the hH_4R, the function of JNJ7777120 as weak partial inverse agonist or even neutral antagonist on [^{35}S]GTPγS binding in the study of Rosethorne and Charlton (2011) implies that at least a small part of JNJ7777120-bound hH_4R molecules stays in an active state, further activating G_i-proteins. A fraction of remaining functional G_i-proteins would not necessarily change β-arrestin recruitment after PTX incubation and might also lead to the congruent concentration-response curves of JNJ7777120-mediated β-arrestin binding with and without PTX (Rosethorne and Charlton, 2011).

Alternatively, Rosethorne and Charlton (2011) could have studied the effects of PTX on histamine- and JNJ7777120-induced ERK activation. Based on the time course, the effects of histamine would be expected to be PTX-sensitive, whereas the effects of JNJ7777120 would be predicted to be PTX-insensitive (Luttrell and Gesty-Palmer, 2010). However, no such control experiments were performed. PTX-sensitive arrestin recruitment has been reported for G_i-coupled receptors (Walters et al., 2009). Intriguingly, the effect of histamine on β-arrestin recruitment in the osteosarcoma cell line is apparently PTX-insensitive, too (Rosethorne and Charlton, 2011). This result is not necessarily in contrast with the possibility that, in the presence of PTX, histamine recruits β-arrestin in a G_i-protein-independent manner differently from a $G\beta\gamma$-dependent manner without PTX, because β-arrestin binding curves may be similar in both cases. But this possibility would imply that the time course of ERK activation by histamine changes after PTX treatment (Luttrell and Gesty-Palmer, 2010; Rosethorne and Charlton, 2011), a consequence that must be checked to exclude insufficient PTX function in the study of Rosethorne and Charlton (2011).

Moreover, one cannot dismiss the possibility that JNJ7777120 stabilizes an hH_4R conformation that enables the receptor to interact even with ADP-ribosylated G_i-protein α-subunits or to preferentially interact with PTX-insensitive G-proteins. Because of slow guanine nucleotide exchange, it can be very difficult or impossible to detect activation of PTX-insensitive G-proteins in the [^{35}S]GTPγS binding assay (Wenzel-Seifert and Seifert, 2000), but activation of PTX-insensitive G-proteins can be assessed more readily with the G-protein photoaffinity labeling/immunoprecipitation technique (Laugwitz et al., 1996). However, such experiments were not conducted in the study of Rosethorne and Charlton (2011). These limitations regarding the G-protein aspect of the study of Rosethorne and Charlton (2011) should be addressed in future studies but do not question the fundamental issue of paradoxical effects of JNJ7777120 at the hH_4R level. We simply have to be cautious with the conclusion that JNJ7777120 recruits β-arrestin without any involvement of active G proteins.

Are H_4R Agonists an Alternative to JNJ7777120 and Thioperamide?

So, if pharmacological effects of JNJ7777120 and thioperamide can no longer be taken for granted as antagonist actions, what about the use of agonists as experimental tools for assessing the pathophysiological role of H_4R? We have recently reviewed the hH_4R agonist literature (Igel et al., 2010). Unfortunately, the situation with agonists is not easier than with H_4R “antagonists.” For example, 5(4)-methylhistamine, originally described as an H_2R agonist (Black et al., 1972), displays selectivity for recombinant hH_4R relative to the other H_xRs (Lim et al., 2005). However, when applied in vivo, the actual concentration of the ligand in a particular organ is unknown, so effects on H_xRs other than the H_4R, specifically the H_2R, cannot be excluded. The anti-inflammatory effects of 5(4)-methylhistamine and JNJ7777120 in a mouse asthma model could be interpreted as agonistic effects of the ligands on the H_4R (Morgan et al., 2007; Neumann et al., 2010), but the effects of 5(4)-methylhistamine could also be mediated via activation of H_2R, exhibiting an established anti-inflammatory role (Hill et al., 1997).

We have described the cyanoguanidine 2-cyano-1-[4-(1*H*-imidazol-4-yl)butyl]-3-[(2-phenylthio)ethyl]guanidine (UR-PI376) as a potent and selective hH_4R agonist (Igel et al., 2009a), but for studies in mouse, rat, and dog, the compound is not useful because of low potency and efficacy (Schnell et al., 2011). The N^G-acylated imidazolylpropylguanidine H_4R agonist N^1-[3-(1*H*-imidazol-4-yl)propyl]-N^2-propionylguanidine (UR-PI294) (Igel et al., 2009b) should also only be used with caution, considering the mechanistically still unexplained and very unusual "superagonism" at recombinant rH_4R, measuring G_i-protein-catalyzed GTP hydrolysis as parameter (Schnell et al., 2011).

Conclusions and Future Studies. Even at the very beginning, the H_4R pharmacology caused headache, as exemplified by contradictory and still unresolved H_1R antagonist effects (Hough, 2001). This initial phase was followed by a relatively headache-free phase in which these intriguing H_1R antagonist effects were put aside and JNJ7777120 rapidly advanced to the status of "standard" H_4R antagonist. And now we have headache, again, because multiple ligands encompassing classic H_4R "antagonists" such as JNJ7777120 and thioperamide, and even agonists show paradoxical, unexpected, unexplained, and complex effects in various systems (Table 1; Figs. 2 and 3).

The recent data from Rosethorne and Charlton (2011) and other groups have important implications for future research in the H_4R field. First, considering the lack of a standard H_4R antagonist, it is necessary to study multiple structurally diverse compounds at multiple H_4R orthologs, assessing multiple G-protein-dependent and -independent parameters, in agonist, antagonist, and inverse agonist modes. There is no way to avoid these painful experimental approaches because even among compounds that are structurally very similar, unexpected pharmacological differences may be uncovered (Schneider et al., 2010). Second, the above-mentioned pharmacological studies have to be accompanied by mechanistic studies aiming at the elucidation of the structural basis for the functional diversity, both with respect to receptors and ligands. This is also not an easy task because the H_4R species orthologs are structurally very different from each other (Lim et al., 2008, 2010; Schnell et al., 2011). In fact, combinations of amino acids and entire receptor regions may account for the different pharmacological properties of H_4R orthologs. Third, crystal structures of the H_4R would be most useful; again, however, structures with multiple ligands and multiple interacting proteins such as G-proteins and arrestins would be required. From all these considerations, it becomes evident that the challenges in the H_4R field are formidable, and presently, it is not clear whether a "standard" H_4R antagonist will ever be identified. But perhaps another strategy will help us out of the dilemma in a relatively short period of time: It is possible that other groups have observed paradoxical effects of H_4R ligands in general and JNJ7777120 in particular as well but have elected not to publish the data so far because they "don't fit" to current, or more correctly, as is outlined in this article, past paradigms. Open and unbiased documentation of H_4R ligand effects in recombinant and native systems will help us understand biased H_4R signaling and the still poorly understood pathophysiological function of the H_4R.

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Authorship Contributions

Participated in research design: Seifert, Dove, and Buschauer.

Contributed new reagents or analytic tools: Dove and Strasser.

Performed data analysis: Seifert, Schneider, Dove, Brunskole, Neumann, Strasser, and Buschauer.

Wrote or contributed to the writing of the manuscript: Seifert, Schneider, Dove, Brunskole, Neumann, Strasser, and Buschauer.

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