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Agonist-Biased Signaling at the Histamine H_4 Receptor: JNJ7777120 Recruits β-Arrestin without Activating G Proteins

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ABSTRACT

The $G_{i/o}$-coupled histamine H_4 receptor is highly expressed in hemopoietic cells and is a promising new target for the treatment of chronic inflammatory diseases. 1-[(5-Chloro-1*H*-indol-2-yl)carbonyl]-4-methyl-piperazine (JNJ7777120) has been described as a selective antagonist at the H_4 receptor and is widely used to characterize the physiological role of the H_4 receptor. We have investigated the pharmacological properties of JNJ7777120 using two distinct downstream signaling measurements, G protein activation and β-arrestin recruitment. The H_4 receptor agonists histamine and clobenpropit, but not JNJ7777120, were able to induce [^{35}S]GTPγS binding in membranes prepared from U2OS-H_4 cells. Thioperamide, a dual H_3/H_4 receptor antagonist, and JNJ7777120 were both able to inhibit the [^{35}S]GTPγS binding induced by clobenpropit. Agonists and antagonists specific for other members of the histamine receptor family had no effect in this assay format. Histamine and clobenpropit increased β-arrestin recruitment to the H_4 receptor in a concentration-dependent manner. This β-arrestin recruitment could be inhibited by preincubation with thioperamide. We were surprised to find that preincubation with the H_4-selective antagonist JNJ7777120 potentiated rather than antagonized the response to a submaximal concentration of clobenpropit. JNJ7777120 treatment alone resulted in an increase in β-arrestin recruitment, which again could be inhibited by preincubation with thioperamide. Schild analysis demonstrated competitive antagonism between thioperamide and both clobenpropit and JNJ7777120. Histamine and clobenpropit had comparable potencies for both [^{35}S]GTPγS binding and β-arrestin recruitment, suggesting little difference in the levels of receptor reserve between the two assays. In conclusion, we have demonstrated that JNJ7777120 recruits β-arrestin to the H_4 receptor, independent of G protein activation.

Introduction

Histamine has many diverse biological roles and exerts its effects via 4 distinct receptor subtypes: H_1, H_2, H_3, and H_4 receptors. The most recent addition to the family, the H_4 receptor, shares a relatively higher degree of homology with the H_3 receptor than either of the H_1 or H_2 receptors (Nakamura et al., 2000; Oda et al., 2000). Unlike the H_1 receptor ($G_{q/11}$) and H_2 receptor (G_s), the H_3 and H_4 receptors couple to $G_{i/o}$, and their activation results in the inhibition of adenylyl cyclase. The expression of the H_4 receptor seems to be limited to the hematopoietic cells, including mast cells (Hofstra et al., 2003), eosinophils (Liu et al., 2001), and T cells (Gutzmer et al., 2009); as such, a role for this receptor in inflammatory responses has been suggested (for review, see Zhang et al., 2007).

Initial investigation into the pharmacology and physiological roles of the H_4 receptor were complicated by the lack of specific ligands at this receptor. The H_4 receptor was found to cross-react with known histamine receptor ligands, most notably those that were previously identified as selective for the H_3 receptor. The H_3 receptor antagonists clobenpropit and clozapine have subsequently been shown to have partial agonist activity at the H_4 receptor (Liu et al., 2001), whereas thioperamide has now been identified as a dual H_3/H_4 receptor antagonist (Gbahou et al., 2006). Many studies investigating the roles of the H_4 receptor have been performed using a combination of these compounds and knockout mice. The identification of JNJ7777120 as a potent and highly selective antagonist at the H_4 receptor marked the advent of more direct investigation into the roles of the H_4 receptor. JNJ7777120 binds to the H_4 receptor with high affinity (K_i, 4 nM) and at least 1000-fold selectivity over the H_1 or H_3 receptors. The antagonist properties of this compound have also been described against histamine in a cAMP-mediated reporter gene assay with a resulting pA_2 of 8.1 (Thurmond et al., 2004).

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ABBREVIATIONS: JNJ7777120, 1-[(5-chloro-1*H*-indol-2-yl) carbonyl]-4-methyl-piperazine; 2-PED, 2-pyridylethylamine dihydrochloride; MEM, minimal essential medium; GPCR, G protein-coupled receptor; HBSS, Hanks' balanced saline solution; BSA, bovine serum albumin; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TBS-T, Tris-buffered saline-Tween 20; ERK, extracellular signal-regulated kinase; U2OS; PathHunter U2OS β-arrestin:EA; U2OS-H_4; PathHunter U2OS β-arrestin:EA-H_4:ProLink.

JNJ7777120 has been used extensively to elucidate the roles of the H_4 receptor in a variety of allergic and inflammatory processes, including chemotaxis of eosinophils (Ling et al., 2004) and mast cells (Thurmond et al., 2004), as well as allergic rhinitis (Takahashi et al., 2009) and allergic airway inflammation (Dunford et al., 2006). Taken together, these data demonstrate that JNJ7777120 has anti-inflammatory properties and suggest that H_4 receptor antagonists may constitute a new class of anti-inflammatory drugs.

The H_4 receptor has previously been shown to couple to multiple signaling pathways, depending on cell background. In recombinant systems, activation of $G_{i/o}$ proteins results in a decrease in adenylyl cyclase activity and hence reduction in levels of cAMP within the cell (Nakamura et al., 2000; Oda et al., 2000). However, in some cell types, such as mouse mast cells, the endogenous H_4 receptor has been shown to couple to Ca^{2+} mobilization, but not to cAMP, in a pertussis toxin-sensitive manner (Hofstra et al., 2003). Furthermore, there is growing evidence that where multiple pathways are activated by the same receptor, certain agonists are able to preferentially activate one pathway over another, displaying collateral efficacy (Kenakin, 2005). This pathway specificity is an important consideration, particularly where ligands are used to characterize the pharmacology and physiology of novel receptors.

In this study, we investigated the ability of several ligands to initiate diverse signaling pathways from the H_4 receptor, comparing G protein-dependent signaling with β-arrestin recruitment that can occur independently of G protein activation (Wei et al., 2003; Lefkowitz and Shenoy, 2005).

Materials and Methods

Histamine Receptor Agonists/Antagonists Used. Histamine, JNJ7777120 [1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methyl-piperazine], triprolidine, mepyramine, ranitidine hydrochloride, clobenpropit dihydrobromide, and thioperamide maleate salt were all purchased from Sigma Ltd. (Poole, UK). 2-Pyridylethylamine dihydrochloride (2-PED) was purchased from Tocris Cookson Ltd. (Bristol, UK). Further information regarding agonists/antagonists appears in Table 1.

Cell Culture. PathHunter U2OS β-arrestin:EA (U2OS) cells (DiscoveRx, Fremont, CA) were maintained in MEM containing L-glutamine supplemented with fetal bovine serum [10% (v/v)], penicillin (100 IU/ml), streptomycin (100 μg/ml), and hygromycin (250 μg/ml) at 37°C, 5% CO_2. For experiments, cells were harvested using trypsin/EDTA and seeded in medium composed as above.

H_4 Receptor Transfection. The PathHunter β-arrestin assay (DiscoveRx) uses enzyme fragment complementation to measure recruitment of β-arrestin to a GPCR after activation. U2OS cells were transfected with the human H_4:ProLink receptor using FuGENE6 according to manufacturer's instructions. They were grown under antibiotic selection [250 μg/ml hygromycin and 500 μg/ml G418 (Geneticin)] to create a "stable pool" of transfected cells. This pool was subsequently single-cell-sorted, and clones were selected that expressed the H_4 receptor at various levels (from here on known as U2OS-H_4 cells). Unless otherwise indicated, the same clonal cell-line was used for all experiments.

TABLE 1
Compounds used in this study
Histamine receptor agonists and antagonists used, with receptor subtype selectivity.

Compound	Receptor Subtype	Mode of Action
Histamine	All	Agonist
2-PED	H_1	Agonist
Triprolidine	H_1	Selective antagonist
Mepyramine	H_1	Selective antagonist
Ranitidine	H_2	Selective antagonist
Clobenpropit	H_3	Selective antagonist
	H_4	Reduced efficacy agonist
Thioperamide	H_3	Antagonist
	H_4	Inverse agonist
JNJ7777120	H_4	Selective antagonist

***β*-Arrestin Recruitment Assay.** U2OS or U2OS-H_4 cells were seeded overnight in white-walled, clear-bottomed, 384-well ViewPlates (PerkinElmer Life and Analytical Sciences, See Green, Buckinghamshire, UK) at 10^4 cells/well in 20 μl of MEM, supplemented as above, and incubated at 37°C, 5% CO_2 overnight to achieve a confluent monolayer. Spent media was removed and replaced with 15 μl of assay buffer (HBSS supplemented with 20 mM HEPES and 0.1% bovine serum albumin). Cells were then stimulated with agonist or vehicle (5 μl) for 2 h at room temperature. All incubations were performed in HBSS supplemented with 20 mM HEPES and 0.1% bovine serum albumin. For antagonist experiments, cells were preincubated with antagonist (5 μl) for 15 min before agonist addition. Flash detection reagent (25 μl) was added, and luminescence was read on the LeadSeeker (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) after a 1-min incubation.

Membrane Preparation. Membranes were prepared from U2OS or U2OS-H_4 cell lines. Cells were grown to approximately 90% confluence, removed from culture flasks using a lifting buffer [10 mM HEPES, 0.9% (w/v) NaCl, and 0.2% (w/v) EDTA, pH 7.4] and cell scraper, pelleted by centrifugation (1500 rpm, 10 min), and resuspended in wash buffer 1 (10 mM HEPES and 10 mM EDTA, pH 7.4). The cell suspension was homogenized with an Ultra-Turrax disperser (5 × 10-s bursts; IKA-Werke GmbH & Co. KG, Staufen, Germany). The resultant homogenate was ultracentrifuged at 48,000*g* for 30 min at 4°C (Avanti J-251; Beckman Coulter, Fullerton, CA), the supernatant was discarded, and the pellet was resuspended. This was repeated to wash, and the final pellet was resuspended in wash buffer 2 (10 mM HEPES and 0.1 mM EDTA, pH 7.4) at a concentration of 3 to 5 mg/ml, as determined by the method of Bradford (1976) using BSA as a standard. Aliquots were flash-frozen and maintained at −80°C until required.

[^{35}S]GTPγS Binding Assay. [^{35}S]GTPγS binding was measured using scintillation proximity assay. All experiments were run at room temperature (~21°C) in buffer [20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, pH 7.4, and 0.01% (w/v) BSA] with 10 μg/ml saponin added. U2OS and U2OS0-H_4 membranes (5 μg) were incubated with 3 μM GDP, 0.5 mg/well wheat-germ agglutinin scintillation proximity assay beads, and a range of histamine receptor agonist and antagonists for 30 min. This initial preincubation was performed to allow the agonist and antagonist to equilibrate before the addition of [^{35}S]GTPγS (0.3 nM), which was followed by a further 60-min incubation. The assay plates were centrifuged before detection of [^{35}S]GTPγS binding using single-photon counting (30 s/well read) on a TopCount scintillation counter (PerkinElmer Life and Analytical Sciences).

[^{3}H]Histamine Binding Assay. [^{3}H]histamine binding assays were performed in 96-deep-well plates in a final volume of 500 μl, using a range of concentrations of [^{3}H]histamine (~1–100 nM) to construct saturation binding curves as described by Dowling and Charlton (2006). Membranes derived from U2OS and U2OS-H_4 cells (15 μg/ml) were incubated in assay binding buffer (50 mM Tris-HCl) at 25°C, with gentle agitation for 60 min. Nonspecific binding was defined using 10 μM unlabeled histamine. After an incubation period, bound and free [^{3}H]histamine were separated by rapid vacuum filtration using a FilterMate Cell Harvester (PerkinElmer Life and Analytical Sciences) onto 96-well GF/C filter plates (Millipore, Watford, UK) precoated with 0.3% polyethylenimine. Plates were rapidly washed three times with ice-cold assay binding buffer. After drying (>4 h), 40 μl of Microscint-20 (PerkinElmer Life and Analytical Sciences) was added to each well, and radioactivity was quantified

using single-photon counting (60 s/well read) on a TopCount microplate scintillation reader (PerkinElmer Life and Analytical Sciences).

Western Blot. U2OS-H_4 cells were seeded overnight in clear 12-well plates at 1.5×10^5 cells/well in 2 ml of MEM, supplemented as above, and incubated at 37°C, 5% CO_2 overnight to achieve a confluent monolayer. On the day of the experiment, cells were washed in assay buffer (HBSS supplemented with 20 mM HEPES and 0.1% bovine serum albumin) and rested in 450 μl of assay buffer at 37°C for 15 min. Cells were then stimulated with agonist or vehicle (50 μl) for between 0 and 60 min at 37°C. Assay buffer was aspirated, and cells were lysed with ice-cold 1× Laemmli buffer containing 1 mM dithiothreitol, scraped, and harvested. Samples were sonicated for 15 to 20 s, boiled for 5 min, and spun for 2 min at 14,000*g*, 4°C. Samples were separated on SDS-polyacrylamide gel electrophoresis at 200 V (constant) for 60 min in MOPS running buffer containing antioxidant. Proteins were then transferred from the gel onto nitrocellulose membrane at 30 V (constant) for 60 min. Membranes were blocked in TBS-T [50 mM Tris base, 150 mM NaCl, pH 7.6, and 0.1% (v/v) Tween 20] containing 5% nonfat dry milk and 1% BSA overnight, at 4°C with gentle agitation. Membranes were washed briefly and incubated with primary antibody [anti-phospho-extracellular signal-regulated kinase (ERK) 1:5000 in TBS-T + 0.1% BSA] for 2 h at room temperature with gentle agitation. After three 5-min washes in TBS-T, membranes were incubated with secondary antibody (IRDye 800CW donkey anti-rabbit IgG, 1:15,000) for 1 h at room temperature with gentle agitation. Membranes were washed three times for 10 min each in TBS-T, and fluorescence was detected using an infrared imaging system (Odyssey; LI-COR Biosciences, Cambridge, UK).

Quantification and Data Analysis. Graphs were fitted to data using Prism software (ver. 4.0; GraphPad Software, San Diego, CA), and results are expressed as the mean ± S.E.M. from at least three separate experiments, unless otherwise stated. Statistical analysis performed using GraphPad Prism (ver. 4.0). Concentration-response data were fitted using a four-parameter logistic equation.

Results

Stimulation of [^{35}S]GTPγS Binding. To investigate G protein-dependent activities at the H_4 receptor, we first determined whether histamine was capable of increasing levels of [^{35}S]GTPγS accumulation in membranes prepared from U2OS cells, which are reported to endogenously express H_1R (Verdonk et al., 2006). In this assay, cells are incubated with a ^{35}S-labeled nonhydrolyzable form of GTP ([^{35}S]GTPγS) that will accumulate after agonist activation of the receptor and give a measurement of G protein activation in the cell.

In membranes prepared from U2OS cells, we were unable to detect any agonistic activity with either histamine or the histamine H_1 agonist 2-PED in (Fig. 1A). To measure G protein-dependent activities at the H_4R, we then monitored histamine-mediated [^{35}S]GTPγS binding in membranes prepared from the U2OS-H_4 cells (stably expressing the H_4: ProLink construct). The K_d for histamine in our cell line was found to be 10.5 nM (Table 3), which correlates well with that reported previously for a HEK-H_4 cell-line (15.3 nM) (Morse et al., 2001). This suggests that the addition of the ProLink to the receptor does not interfere with agonist binding to the receptor.

Histamine and clobenpropit, but not JNJ7777120, were both able to induce [^{35}S]GTPγS binding in membranes prepared from U2OS-H_4 cells, with pEC_{50} values of 7.8 ± 0.2 and 7.1 ± 0.2, respectively. In addition, we demonstrated that this assay was capable of detecting constitutive activity, because thioperamide treatment resulted in a reduction in the basal levels of [^{35}S]GTPγS binding (Fig. 1B, Table 2). In contrast, triprolidine had no effect on levels of either basal or histamine stimulated [^{35}S]GTPγS binding in the U2OS-H_4 cells (data not shown).

Thioperamide and JNJ777120 were also both able to inhibit the H_4 receptor-mediated [^{35}S]GTPγS binding induced by clobenpropit (Fig. 1C) with pIC_{50} values of 6.3 ± 0.04 and 6.7 ± 0.19, respectively. Again, we saw a decrease in [^{35}S]GTPγS binding below basal levels with thioperamide, demonstrating that this compound is an inverse agonist in this assay format.

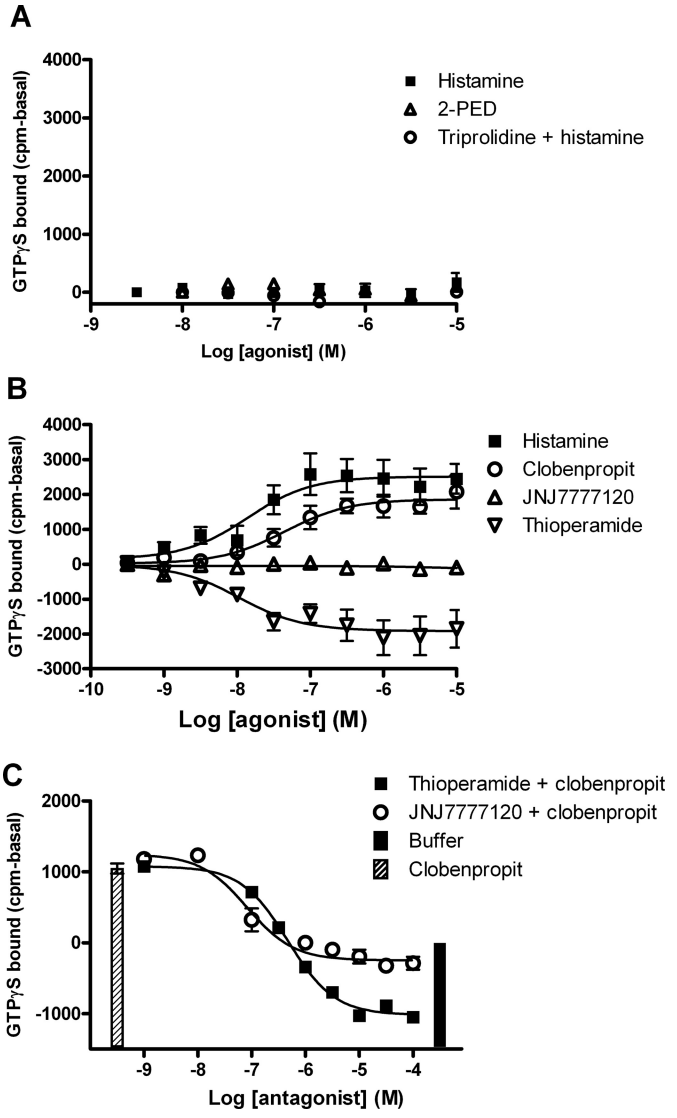

Fig. 1. Receptor-mediated [^{35}S]GTPγS binding in membranes prepared from naive U2OS cells (A) or U2OS-H_4 cells (B and C). A, concentration-dependent increases in [^{35}S]GTPγS binding were assessed after a 1-h stimulation with the indicated concentrations of histamine, 2-PED, or triprolidine plus a single concentration of histamine (100 μM). Concentration-dependent increases in [^{35}S]GTPγS binding were assessed after a 1-h stimulation with the indicated concentrations of histamine, clobenpropit, thioperamide, or JNJ7777120 (B) or JNJ7777120 and thioperamide plus a single concentration of clobenpropit (300 nM) (C). For each individual experiment, data have been normalized to the [^{35}S]GTPγS binding detected after assay buffer addition alone, and are expressed as means ± S.E.M. for at least three independent experiments.

Recruitment of β-Arrestin to the H_4 Receptor. After receptor activation and phosphorylation, β-arrestins are recruited to the active receptor to initiate signal termination via endocytosis of the receptor and uncoupling of the receptor from G protein (Lohse et al., 1990; Pitcher et al., 1992). In addition to this role, it has also been demonstrated that the association of β-arrestin is involved in G-protein independent signaling, such as ERK1/2 activation (Shenoy et al., 2006; Dewire et al., 2007). The PathHunter β-arrestin assay (DiscoveRx) uses β-galactosidase enzyme fragment complementation to measure recruitment of β-arrestin to a GPCR after activation (for details, see DeWire et al., 2007; Olson and Eglen, 2007).

The parental U2OS cell-line was tested in this assay format to confirm that the assay was specific for coupling between the transfected β-arrestin:EA and GPCR:ProLink tags. No β-arrestin recruitment could be detected in cells where the β-arrestin:EA, but no ProLink-tagged receptor, was present (Fig. 2a).

After transfection and clonal selection of the H_4:ProLink receptor, a range of histamine receptor agonists were tested in the β-arrestin assay to determine their ability to recruit β-arrestin to the H_4 receptor. Histamine and clobenpropit, but not the H_1 receptor-selective 2-PED, were able to induce β-arrestin recruitment to the H_4 receptor (Fig. 2A). Histamine- and clobenpropit-mediated β-arrestin recruitment occurred in a concentration-dependent manner (Fig. 2B), with pEC_{50} values of 7.3 ± 0.1 and 7.1 ± 0.1, respectively. EC_{80} concentrations of agonists were used to demonstrate that the β-arrestin recruitment observed could be inhibited by preincubation with thioperamide (Fig. 2C). In contrast, preincubation with JNJ7777120 did not antagonize the histamine response and moderately potentiated the response to a submaximal concentration of clobenpropit where the system was not already saturated (Fig. 2D). JNJ7777120 treatment alone resulted in a concentration-dependent increase in β-arrestin recruitment (pEC_{50}, 7.6 ± 0.1; Fig. 2E) that could be inhibited with pretreatment of the cells with thioperamide (pIC_{50}, 6.4 ± 0.2) but not the H_1-selective antagonist triprolidine. Thioperamide was not able to decrease β-arrestin recruitment below basal levels, demonstrating that this compound is a neutral antagonist in this assay format (Fig. 2F). It has previously been demonstrated that the complementation between the ProLink and EA tags is reversible, making it possible to detect inverse agonist activity in this assay system (Mcguinness et al., 2009). This would imply that although we do not observe constitutive recruitment of β-arrestin to the H_4 receptor in this cell line, the assay system itself is capable of measuring inverse agonism.

To further ensure the JNJ7777120 agonist activity was due to a specific interaction with the H_4 receptor, Schild plots for the antagonist thioperamide were compared against JNJ7777120 and clobenpropit concentration-response curves. Thioperamide produced parallel rightward shifts of both clobenpropit and JNJ7777120 concentration-response curves, yielding pA_2 values of 6.95 ± 0.76 and 6.95 ± 0.62, respectively, and Schild slopes of 0.99 and 0.97, respectively (Fig. 3). This suggests that thioperamide is a competitive antagonist against both JNJ7777120 and clobenpropit.

To determine whether the level of receptor expression was responsible for the agonistic properties of JNJ7777120 in the β-arrestin recruitment assay, a range of clonal cell lines was selected that expressed different levels of receptor, assessed by using [^{3}H]histamine saturation binding (Table 3). These cell lines were then treated with the full agonist histamine and JNJ7777120 to determine levels of β-arrestin recruitment (Fig. 4). The maximal response observed in each cell line increased linearly with receptor expression (Fig. 5), but there was no change in the EC_{50} for either histamine or JNJ7777120 (Table 3; $P > 0.1$ by one-way analysis of variance), suggesting little receptor reserve in this system.

β-Arrestin recruitment does not require active G protein to be recruited to the receptor, but rather serves to uncouple the G protein from the activated receptor and hence initiate signal termination (Lohse et al., 1990; Pitcher et al., 1992). To determine whether the β-arrestin recruitment observed in this study was dependent upon activation of G proteins, cells were treated with agonists after prior incubation with pertussis toxin to inactivate $G_{i/o}$ proteins. Pretreatment for 20 h with 200 ng/ml. Pertussis toxin had no effect on histamine- or JNJ7777120-mediated β-arrestin recruitment (Fig. 6).

Measurement of ERK Phosphorylation. One of the downstream consequences of β-arrestin recruitment is the scaffolding of the mitogen-activated protein kinase (MAPK) complex, which results in ERK phosphorylation and activation (Shenoy et al., 2006; DeWire et al., 2007). To determine whether the JNJ7777120-mediated recruitment of β-arrestin translated into a functional ERK response, ERK

TABLE 2

Efficacy and potency of agonists in different assay formats

Histamine, JNJ7777120, clobenpropit, and thioperamide efficacy and potency in different assay formats using the U2OS-H_4 cell-line. The efficacy of each agonist is expressed as a % of the maximal response to histamine. Data are shown as means ± S.E.M. for at least three independent experiments.

	GTPγS		β-Arrestin	
	pEC_{50}	Efficacy	pEC_{50}	Efficacy
		%		%
Histamine	6.2 ± 0.2	100	7.3 ± 0.1	100
Clobenpropit	7.4 ± 0.2	84.1 ± 9.7	7.1 ± 0.1	86.2 ± 3.9
JNJ7777120		−5.5 ± 2.8	7.9 ± 0.2	64.3 ± 5.1
+ 300 nM Clobenpropit			7.4 ± 0.1	68 ± 12.9
+ 1 μM Clobenpropit	6.9 ± 0.2	−6.0 ± 1.9		
+ 10 μM Histamine			5.6 ± 0.0	119 ± 0.0
Thioperamide	8.0 ± 0.3	−47.3 ± 4.1	N.E.	N.E.
+ 100 nM Histamine			8.0 ± 0.02	−6.7 ± 3.3
+ 100 nM JNJ7777120			6.4 ± 0.1	−6.7 ± 2.4
+ 300 nM Clobenpropit	6.3 ± 0.1	−21.5 ± 1.1	7.6 ± 0.2	−9.5 ± 1.3

N.E., no effect.

phosphorylation was monitored by Western Blot after treatment of cells with a maximal (100 μM) concentration of either JNJ7777120 or histamine for between 0 and 60 min. Treatment with either agonist resulted in ERK phosphorylation in a time-dependent manner (Fig. 7). The magnitude of ERK phosphorylation was similar in both compounds, but the time courses were very different. Histamine induced a transient phosphorylation that peaked at 5 min and returned to basal levels within 30 min. In contrast, JNJ7777120 did not stimulate any appreciable ERK phosphorylation over the first 10 min but then generated a much more prolonged pERK response that peaked at 30 min and was still elevated after 60 min.

Discussion

The H_4 receptor has been shown to be largely expressed in hemopoietic cells and to possess the ability to modulate eosinophil migration and selective recruitment of mast cells, leading to amplification of histamine-mediated immune responses and eventually to chronic inflammation. The discovery of this role in inflammation has led to the proposal that H_4 receptor antagonists may possess anti-inflammatory properties.

JNJ7777120 has been described previously as a novel antagonist at the H_4 receptor with a K_i of 4 nM. It has been characterized in cAMP-cAMP response element reporter assays, where it acts as a competitive antagonist, producing parallel rightward shift of histamine concentration-response curves with increasing concentrations. JNJ7777120 was shown to have a pA_2 equal to 8.1 and demonstrated good selectivity with little or no affinity for over 50 other targets (Thurmond et al., 2004). In addition to studies in recombinant cell systems, JNJ7777120 has been shown to exhibit functional antagonism of Ca^{2+} mobilization and chemotaxis to histamine in mast cells in vitro and in vivo (Jablonowski et al., 2003; Thurmond et al., 2004). We have demonstrated that JNJ7777120 is a biased agonist at the human H_4 receptor, acting as a neutral antagonist for [^{35}S]GTPγS binding and a partial agonist for β-arrestin recruitment.

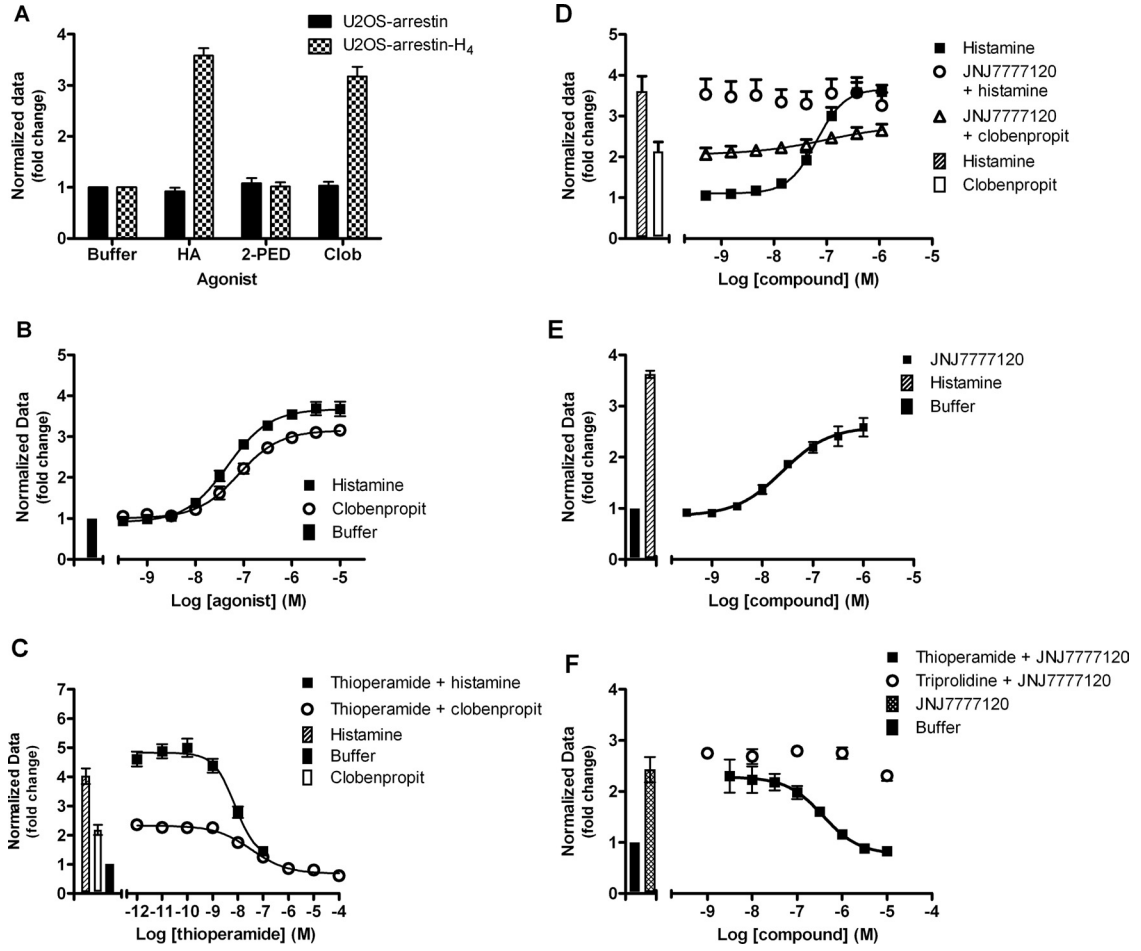

Fig. 2. Receptor-mediated β-arrestin recruitment in U2OS and U2OS-H_4 cells. A, treatment of U2OS or U2OS-H_4 cells with maximal concentration (100 μM) of each agonist for 2 h. B, concentration-dependent increases in β-arrestin recruitment were assessed after a 2-h stimulation with the indicated concentrations of histamine or clobenpropit. Levels of β-arrestin recruitment were monitored after a 15-min preincubation with indicated concentrations of thioperamide followed by a 2-h incubation with a single, EC_{80} concentration of either histamine (300 nM) or clobenpropit (1 μM) (C) or β-arrestin recruitment was assessed after a 2-h stimulation with the indicated concentrations of histamine or preincubation for 30 min with indicated concentrations of JNJ7777120 followed by a 2-h incubation with a single concentration of either histamine (maxima, 10 μM) or clobenpropit (submaximal: 300 nM) (D). E, concentration-dependent increases in β-arrestin recruitment were assessed after a 2-h stimulation with the indicated concentrations of JNJ7777120. F, levels of β-arrestin recruitment were monitored after a 15-min preincubation with indicated concentrations of thioperamide or triprolidine, followed by a 2-h incubation with a single, EC_{80} concentration of JNJ7777120 (100 nM). For each individual experiment, data have been normalized to the β-arrestin recruitment detected after assay buffer addition alone and are expressed as means ± S.E.M. for at least three independent experiments.

The phenomenon of agonist-biased signaling has been described for a number of different systems (for reviews, see Rajagopal et al., 2010; Kenakin and Miller, 2010), both in vitro (Berg et al., 1998; Cordeaux et al., 2000, 2001; Gay et al., 2004; Lane et al., 2007) and in vivo in the case of the nicotinic acid receptor (GPR109a) (Semple et al., 2008). This has been attributed to the existence of multiple active conformations, each agonist being able to stabilize a subtly different state that may preferentially couple to one pathway over another (Kenakin, 2001; Bokoch et al., 2010). It is, however, very important to rule out other potential explanations for the observation of biased signaling before speculating that different receptor conformations may be responsible.

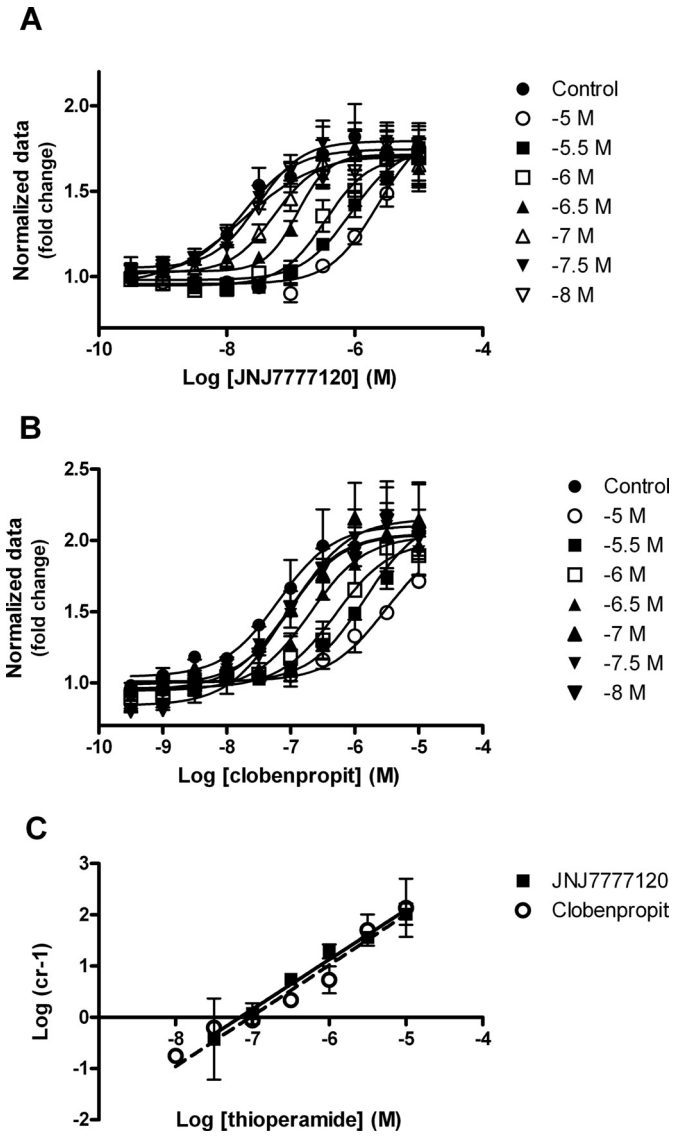

Fig. 3. Levels of β-arrestin recruitment were monitored after a 15-min preincubation with indicated concentrations of thioperamide, followed by a 2-h incubation with concentration-response curves to JNJ7777120 (A) or clobenpropit (B). C, data were used to calculate dose ratios and Schild analysis performed using Prism software. For each individual experiment, data have been normalized to the β-arrestin recruitment detected after assay buffer addition alone and are expressed as means ± S.E.M. for at least three independent experiments.

First, and most importantly, it is critical to ensure that differences in agonist efficacy are not responsible. For example, in a system with poor coupling efficiency, a low-efficacy agonist may seem to be a neutral antagonist. If, however, the receptor reserve is increased, a low-efficacy agonist may become a full agonist in that particular system (Baker et al., 2002; Kenakin, 2009). For this reason, it is important to consider receptor reserve in each pathway examined.

We observed that clobenpropit and histamine display similar potency and efficacy in both the [^{35}S]GTPγS binding assay and β-arrestin recruitment assay, indicating there is little difference in the efficiency of coupling of the two different signaling pathways (Table 2). If JNJ7777120 were truly a partial agonist that failed to score in the [^{35}S]GTPγS assay, we would also expect it to appear inactive in the similarly coupled β-arrestin recruitment assay. In addition, previous work using the highly amplified cAMP-cAMP response element transcription factor assay has demonstrated that JNJ7777120 is a neutral antagonist in an assay system that is capable of measuring inverse agonism (Lim et al., 2005).

To further test whether the high level of maximal JNJ7777120 agonist responses in the β-arrestin recruitment assay were an artifact of overexpressing the H_4 receptor in the U2OS cells, four different clonal cell lines were tested in a radioligand saturation binding assay, and the β-arrestin recruitment assay. In all of the clones tested, there was a linear correlation between receptor expression and maximal activation by both histamine and JNJ7777120, but no shift in the pEC_{50} of either compound. The absence of receptor reserve is probably a result of the fixed one-to-one stoichiometry between receptor and β-arrestin in this assay system, as has recently been suggested by Waterfield et al. (2009). It is therefore unlikely that the agonist activity of JNJ7777120 in the β-arrestin recruitment assay is an artifact of receptor overexpression in

TABLE 3

H_4 receptor expression and agonist efficacy and potency in cell- ines expressing different levels of the H_4 receptor

[^{3}H]Histamine binding was used to determine the level of H_4 receptor expression in a number of U2OS-H_4 cell lines. These cell lines were then used to characterize the efficacy and potency of histamine and JNJ7777120. Efficacy of each agonist is expressed as a percentage of the maximal response to that ligand observed in the highest expressing clone (ER1J). In addition, the percentage efficacy for JNJ7777120 compared with the histamine response in that clone is shown in parentheses. Data are shown as means ± S.E.M. for three independent experiments.

	[^{3}H]-histamine		Histamine		JNJ7777120	
	B_{max}	K_d	pEC_{50}	Efficacy	pEC_{50}	Efficacy
	pmol/mg	*nM*		%		%
U2OS-H_4_ER1J	2.2 ± 0.2	10.5 ± 2.3	7.5 ± 0.07	75.7	7.9 ± 0.10	100 (66.0)
U2OS-H_4_ER1L	1.3 ± 0.1	11.6 ± 3.7	7.7 ± 0.10	42.9	8.1 ± 0.10	57.2 (75.9)
U2OS-H_4_ER1B	1.1 ± 0.2	10.8 ± 3.4	7.5 ± 0.05	27.8	8.0 ± 0.05	37.2 (64.8)
U2OS-H_4_ER1K	0.3 ± 0.1	22.2 ± 13.0	7.7 ± 0.08	3.1	7.9 ± 0.26	4.2 (97.0)

these cells. Although it may not be indicative of endogenous receptor-β-arrestin interactions, this lack of reserve might be expected for this type of assay system.

It is important to also ensure that the agonists are acting at a single receptor. If the ligand is not fully selective, it may activate other pathways via a different receptor, apparently displaying biased signaling. We have taken several steps to prove that JNJ7777120 is not acting as an agonist at a different receptor to recruit β-arrestin. First, we tested a range of ligands with different histamine receptor selectivity to show that the signaling we observed was solely via the H_4 receptor. Second, the assay employed to measure β-arrestin recruitment uses enzyme complementation, so only recruitment of β-arrestin to the tagged receptor will result in reconstitution of the enzyme activity. This means that the signal is very specific for the H_4 receptor. Finally, we performed Schild plots with the H_4 receptor antagonist thioperamide to demonstrate that the resulting pA_2 value against JNJ7777120 was the same as that against clobenpropit, strongly suggesting they are acting as the same receptor site.

It has recently been shown that differences in dissociation rates between ligands can also lead to the misinterpretation of agonist-biased signaling, particularly if one of the assay measures is rapid and the other is slower (Vauquelin and Charlton, 2010). Although the lack of kinetic data on these compounds means this explanation cannot

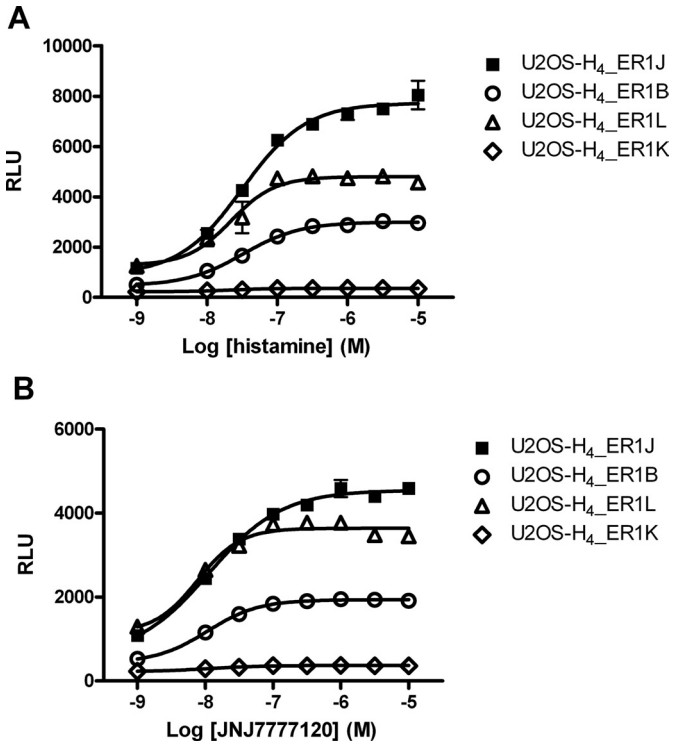

Fig. 4. Receptor-mediated β-arrestin recruitment in U2OS cells expressing different levels of the H_4:ProLink receptor. Concentration-dependent increases in β-arrestin recruitment were assessed in four separate clones of U2OS-H_4 cells after a 2-h stimulation with the indicated concentrations of histamine (A) or JNJ7777120 (B). For each individual experiment, data have been normalized to the β-arrestin recruitment detected after assay buffer addition alone and are expressed as means ± S.E.M. for at least three independent experiments.

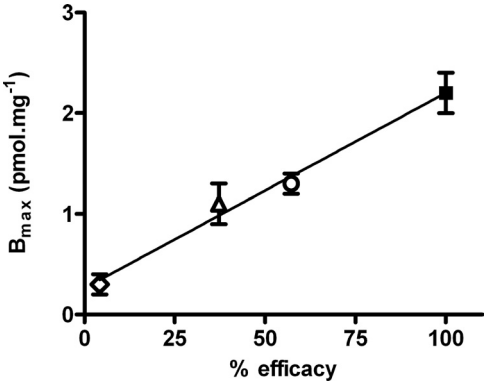

Fig. 5. Correlation between H_4 receptor expression (in picomoles per milligram) and maximal histamine-induced recruitment of β-arrestin (as a percentage of that observed in the highest expressing cell line) in four different clonal U2OS-H_4 cell lines.

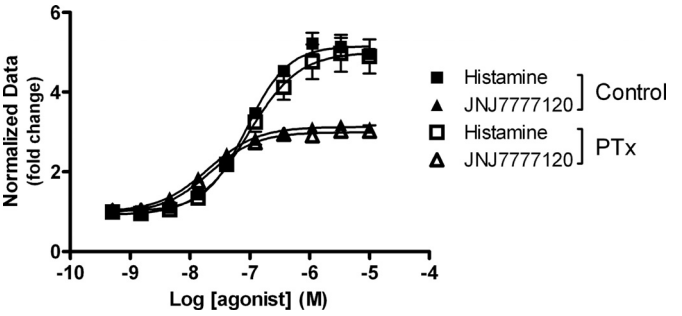

Fig. 6. Receptor-mediated β-arrestin recruitment in U2OS-H_4 cells after pertussis toxin treatment. Concentration-dependent increases in β-arrestin recruitment were assessed after a 2-h stimulation with the indicated concentrations of histamine or JNJ7777120, 20 h after treatment with pertussis toxin (200 ng/ml). For each individual experiment, data have been normalized to the β-arrestin recruitment detected after assay buffer addition alone and are expressed as means ± S.E.M. for at least three independent experiments.

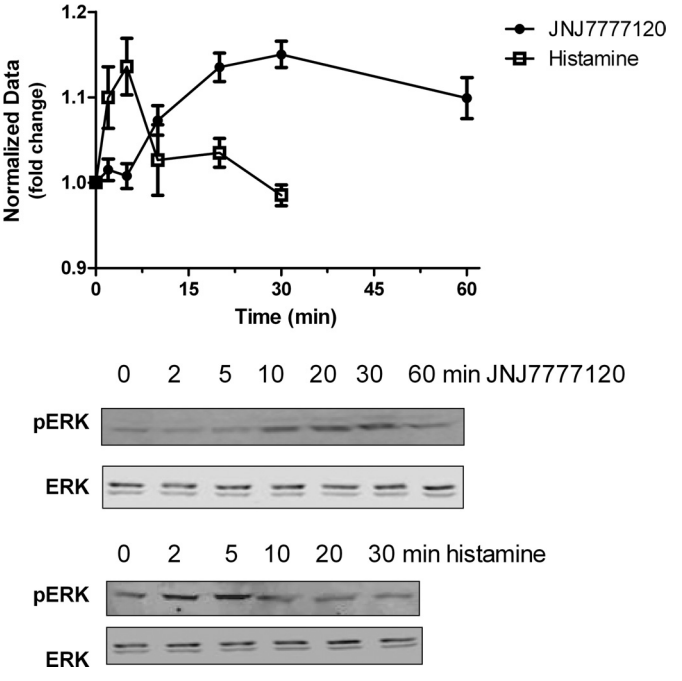

Fig. 7. Receptor-mediated ERK phosphorylation. Time-dependent increases in the levels of phosphorylated ERK were assessed by Western blot after treatment of U2OS-H_4 cells with a maximal (100 μM) concentration of JNJ7777120 or histamine. For each individual experiment, data have been normalized to the ERK phosphorylation detected after addition of vehicle alone and are expressed as means ± S.E.M. for at least three independent experiments.

be completely excluded, the long nature of both assay measures means that hemiequilibrium is unlikely to be an issue in this study.

Having ruled out many of the common artifacts that may complicate the interpretation of biased signaling, we conclude that JNJ7777120 is likely to stabilize an alternative active conformation of the H_4R that is able to initiate β-arrestin recruitment but not G protein activation. By examining ERK phosphorylation in these cells downstream of JNJ7777120, we have also been able to demonstrate that this recruitment of β-arrestin translates into a functional response. The time-course of JNJ7777120 activation of ERK was delayed compared with that of histamine, a peak response being observed between 20 and 30 min rather than the rapid and transient response observed with histamine. This observation is consistent with previous reports showing that G protein-mediated ERK phosphorylation is fast and short-lived, whereas β-arrestin-dependent pERK responses are slower and more prolonged (Ahn et al., 2004).

JNJ7777120 has been used extensively in vitro and in vivo to elucidate H_4 receptor biology. We have demonstrated that although JNJ7777120 is an antagonist with regard to G protein-dependent signaling, it acts as an agonist in a non–G protein-dependent manner to recruit β-arrestin to the receptor, as demonstrated by the lack of sensitivity to pertussis toxin treatment. In addition to its traditional role in desensitization, β-arrestin has been shown to be involved in the scaffolding and regulation of G protein-independent signaling pathways, such as ERK1/2, c-Jun NH_2-terminal kinase, and c-Raf-1 (Lefkowitz and Shenoy, 2005). For this reason, the agonist properties of JNJ7777120 could potentially complicate interpretation of experimental data, especially from in vivo work, and lead to misleading conclusions regarding the role of the H_4R. This work has demonstrated that the properties of JNJ7777120 are dependent upon the system studied and highlights the need to consider more than one downstream signaling pathway when evaluating compounds for efficacy at GPCRs.

Authorship Contributions

Participated in research design: Rosethorne and Charlton.
Conducted experiments: Rosethorne.
Performed data analysis: Rosethorne.
Wrote or contributed to the writing of the manuscript: Rosethorne and Charlton.

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