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G Protein-Dependent Pharmacology of Histamine H_3 Receptor Ligands: Evidence for Heterogeneous Active State Receptor Conformations

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ABSTRACT

Previously reported pharmacological studies using the imidazole-containing histamine H_3 receptor ligands GT-2331 (Ciprallisant) and proxyfan resulted in a range of classifications (antagonist, agonist, and protean) for these compounds. We examined the role that the signaling system, with particular emphasis on the type of G protein, had on the pharmacology observed for H_3 ligands. Ligands were assessed using assays measuring neurotransmitter release, cAMP, and guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding. Whereas clobenpropit and ciproxifan were consistently antagonists, GT-2331, proxyfan, and imetit exhibited differential activity. Although GT-2331 and proxyfan exhibited little agonist activity in neurotransmitter release assays, both demonstrated full agonism relative to (*R*)-α-methylhistamine in cAMP assays. In [^{35}S]GTPγS binding assays, GT-2331 and proxyfan demonstrated partial agonism. Imetit showed full agonism in most assays, but it was slightly less efficacious in a neurotransmitter release assay and in [^{35}S]GTPγS binding at the human H_3 receptor. To further examine these ligands, we coexpressed Gα16 or chimeric Gαq/i_5 in human embryonic kidney cells expressing the human H_3 receptor and assayed intracellular calcium and cAMP levels. GT-2331, proxyfan, and imetit demonstrated full agonism in all assays of cAMP activity. However, in cells expressing Gα16, they exhibited minimal agonism in calcium mobilization assays, whereas imetit showed partial agonism. When Gαq/i_5 was used, the activity of both GT-2331 and proxyfan increased, whereas imetit became a full agonist. These results demonstrate that GT-2331 and proxyfan's differential pharmacology at the H_3 receptor depends on the type of G protein used and provide indirect evidence for differential ligand-bound active states that mediate signaling by the H_3 receptor.

The histamine H_3 receptor is a member of the large superfamily of G protein-coupled receptors (GPCRs) that are characterized by the presence of seven putative transmembrane-spanning domains. Since its identification in 1983 (Arrang et al., 1983), the histamine H_3 receptor has been shown to regulate presynaptic release of a variety of neurotransmitters, including histamine, dopamine, noradrenaline, serotonin, and acetylcholine (Blandina et al., 1998). Hence, this receptor has been postulated to be a good target for drug discovery for a variety of indications. A wide range of structurally diverse ligands has been synthesized (for overview, see Leurs et al., 2005) as potential drug candidates or as pharmacological tools.

The initial cloning of the H_3 receptor (Lovenberg et al., 1999) and subsequent molecular biological and pharmacological studies have revealed species differences and multiple splice variants of the H_3 receptor (Ligneau et al., 2000; Tardivel-Lacombe et al., 2000; Cogé et al., 2001; Drutel et al., 2001; Stark et al., 2001, Wellendorph et al., 2002; Chen et al., 2003; Yao et al., 2003). Although many of the apparent disparities in H_3 receptor ligand pharmacology that have been reported in the literature may be attributed to species and/or splice variant differences (for overview, see Hancock et al., 2003), these explanations have been insufficient to account for pharmacological discrepancies observed with certain H_3 ligands. For example, initial pharmacological characterization of two imidazole-containing ligands, GT-2331 (Cipral-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; AM, acetoxymethyl ester; NAMH, *N*-α-methylhistamine; [^{35}S]GTPγS, 5′-*O* -(3-[^{35}S]thio) triphosphate; RAMH, (*R*)-α-methylhistamine; HEK, human embryonic kidney; ANOVA, analysis of variance; EFS, electrical field-stimulated; GPI, guinea pig ileum; PEI, polyethylenimine; FLIPR, fluorescent imaging plate reader; FSK, forskolin.

isant) and proxyfan (Hüls et al., 1996; Tedford et al., 1998), indicated that these compounds acted as antagonists at the histamine H_3 receptor. Subsequent studies revealed more complex pharmacology. GT-2331 and proxyfan were shown to possess agonist activity at recombinantly expressed human H_3 receptors (Wulff et al., 2002). Other studies characterized proxyfan as either a neutral antagonist (Morisset et al., 2000; Rouleau et al., 2002) or a partial agonist (Liedtke et al., 2003). More recently, a publication by Gbahou et al. (2003) indicated that proxyfan acted as a protean agonist at the rodent receptor, displaying characteristics ranging from inverse agonism to agonism, depending on the system examined. Our studies were aimed at further characterizing this differential pharmacology and examining the role that the G protein had in the pharmacology displayed by these compounds. To do this, a variety of functional assays were performed where signaling through either endogenous G protein or cotransfected Gα subunits was determined. The pharmacology of GT-2331 and proxyfan, relative to classic H_3 agonists and antagonists, was compared across these assay systems. Profound efficacy differences and some potency differences were found that were dependent on the signaling system and type of G protein used. Although these results provide evidence for differential H_3 coupling through different signaling systems and G proteins, they are also suggestive of alternative ligand-bound active state conformations for the H_3 receptor that may arise by ligand-selection or ligand-induction (Kenakin, 2003; Kobilka, 2005). The presence of alternative ligand-bound conformations has been well established for some other types of GPCRs (for reviews, see Kenakin, 2001; Kenakin, 2003), and this topic is discussed further in the context of our experimental results.

Materials and Methods

Materials. Male Sprague-Dawley rats and Hartley guinea pigs (both weighing 150–200 g upon arrival) were purchased from Charles River Breeding Laboratories (Portage, MI) and were housed in facilities at Abbott Laboratories (Abbott Park, IL). The facilities were approved by and all studies were carried out in accordance with guidelines outlined by the Animal Welfare Act, the Association for Assessment and Accreditation of Laboratory Animal Care, and the Institutional Animal Care and Use Committee of Abbott Laboratories. Rat tissue was obtained from Pel-Freez (Rogers, AK), and human tissue was obtained from Analytical Biological Services Inc. (Wilmington, DE). Fluo-4-acetoxymethyl ester (Fluo-4-AM), a calcium indicator dye, was purchased from Molecular Probes (Eugene, OR). Measurements of cAMP levels were determined using either scintillation proximity assays from Amersham Biosciences Inc. (Piscataway, NJ) or [^{125}I] Flashplate assays from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{3}H]*N*-α-Methylhistamine ([^{3}H]NAMH), [^{35}S]GTPγS, and [^{3}H]histidine were also from PerkinElmer Life and Analytical Sciences. GT-2331, proxyfan, and ciproxifan were synthesized at Abbott Laboratories. (*R*)-α-Methylhistamine (RAMH) and clobenpropit were from Tocris Cookson Inc. (Ellisville, MO). LipofectAMINE 2000 and all cell culture reagents were purchased from Invitrogen (Carlsbad, CA). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Membrane Preparations and Radioligand Binding Assays. Cloning of the histamine H_3 receptors and creation of C6 or HEK293 cell lines expressing the human or rat full-length H_3 receptor [hH_3(445) or rH_3(445), respectively; for proposed nomenclature, see Hancock et al., 2003] were described previously (Esbenshade et al., 2003). Membranes from these cells, from rat cerebral cortex or from human cortex were prepared by homogenization on ice in 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM EDTA (TE buffer), 1 mM benzamidine, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. The homogenate was centrifuged at 40,000*g* for 20 min at 4°C. This step was repeated, and the resulting pellet was resuspended in TE buffer. Aliquots were frozen at −70°C until needed. On the day of assay, membranes were thawed and diluted with TE buffer. For saturation assays, various concentrations of [^{3}H]NAMH were incubated with cell membranes either alone or in the presence of 10 μM thioperamide at 25°C for 30 min, and B_{max} and K_d values were determined by nonlinear regression analysis of binding isotherms using GraphPad Prism (GraphPad Software Inc., San Diego, CA). For competition assays, 0.5 to 1.0 nM [^{3}H]NAMH was incubated with cell membranes either alone or in the presence of competing histaminergic ligands at 25°C for 30 min. Nonspecific binding was defined with 10 μM thioperamide. Radioligand binding was terminated by filtration onto 0.3% polyethyleneimine (PEI)-pretreated GF/B filters followed by thorough rinsing with 50 mM Tris-HCl buffer, pH 7.4. The amount of bound radiolabel was determined by liquid scintillation counting. Data analysis was performed as described by Esbenshade and Hancock (2000), with pK_i values determined using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Statistical analyses of pK_i data were performed using a one-way ANOVA followed by Tukey's pairwise comparison.

Electric Field-Stimulated Guinea Pig Ileum Neurotransmitter Release Assays. The method of Trzeciakowski (1987) was used to examine the functional responses of histamine H_3 receptor ligands on electric field-stimulated (EFS) guinea pig ileum (GPI) contractions. A 20-cm section of ileum, obtained approximately 10 cm proximal to the ileocecal junction, was removed from young male guinea pigs (150–250 g) and sectioned into 2-cm tube segments, cleaned, and placed in warm (37°C) Krebs-Henseleit bicarbonate buffer containing 2.5 mM $CaCl_2$, 100 nM pyrilamine, and 10 μM cimetidine. One end of the segment was then mounted onto a stationary rod containing parallel platinum electrodes aligned on each side of the tissue, and the other end was connected to a Grass FT03 transducer at a basal preload tension of 1 g. After a 30-min equilibrium period in heated (37°C) 10-ml organ baths, the tissues were electrically stimulated using a train of 0.1 per second, train duration 1000 ms, frequency 10 Hz, duration 0.5 ms, and supramaximal voltage 10 V, and rinsed every 10 min for 1 h. The intensity of the stimulus was then decreased every 5 min, initially by 2 V (smaller decrements in voltage as the threshold was approached) until the threshold voltage for EFS contraction could be established. The experiment was then run at a test voltage average of 6.5 to 7.5 V. This submaximal voltage protocol evoked a contractile twitch, 2.34 ± 0.12 g, $n = 21$. The tissues were stimulated for an additional 30 min before drug addition. Agonists were tested by the noncumulative addition of semilogarithmically increasing concentrations to the baths. RAMH was used as a reference standard H_3 agonist. RAMH was introduced into the tissue bath chamber, allowed 3 min of contact time, and rinsed. A 20-min interval was used and the cycle was repeated. RAMH reduced the stimulated twitch response over a concentration range of 0.3 nM to 3 μM with a $pEC_{50} = 7.46 \pm 0.09$ and 72.6% reduction of the maximum response. Each tissue preparation was only used for one response curve since repetitive applications of RAMH desensitize the H_3 receptor (Perez-García et al., 1998).

Antagonists were preincubated for 60 min, followed by a rinse, and a readdition of the antagonist for another 30 min. RAMH was then introduced in the presence of the test antagonist, allowed 3 to 4 min to yield a full effect, rinsed, and the antagonist was readded to the tissue bath chamber. This noncumulative protocol was repeated every 30 min until the complete response curve was completed.

Data were recorded and analyzed using PowerLab/80 analog to digital converter and Chart software (ADInstruments Pty Ltd., Castle Hill, Australia). Data were measured in grams as the difference between the baseline (1 g) and maximum effect. The concentration of the agonist necessary to cause a 50% inhibition in the EFS contraction (EC_{50}) was calculated using an Excel-based program, AGANTG

(Zielinski and Buckner, 1998). The potency of the antagonists (pA_2) to inhibit the RAMH response was calculated according to the method of Schild (1947) using GraphPad Prism.

Rat Cerebral Cortical Histamine Release Assay. Synaptosomes were prepared using freshly isolated rat cerebral cortex tissue (Esbenshade et al., 2003) and were incubated with 1.2 μM [^{3}H]histidine for 30 min at 37°C under a constant stream of O_2, CO_2 (95:5) in a modified Krebs-Ringer bicarbonate buffer (1.2 mM KH_2PO_4, 0.67 mM $MgSO_4$, 0.8 mM KCl, 2.6 mM $CaCl_2$, 27.5 mM $NaHCO_3$, 120 mM NaCl, and 10 mM D-glucose) to allow uptake of [^{3}H]histidine and conversion to [^{3}H]histamine. After extensive washing to remove unincorporated [^{3}H]histidine, the synaptosomes were resuspended in modified Krebs-Ringer bicarbonate buffer, and aliquots (containing approximately 2 mg of protein) were added to microcentrifuge tubes containing either agonist alone or in concert with antagonist. The mixture was incubated for 2 min at 37°C in the presence of 5% CO_2 to allow the ligand to bind before the addition of potassium (final concentration, 15 mM). The samples were incubated for an additional 2 min and subsequently placed on ice and centrifuged (4°C) at 20,000*g* for 20 min. The supernatant was removed, and the amount of [^{3}H]histamine released was determined after chromatography of the supernatant on Amberlite CG-50 resin, as described previously (Garbarg et al., 1981). Basal release (in buffer without additional potassium) values were subtracted from each sample, and the data were expressed as a percentage of the maximum potassium-stimulated [^{3}H]histamine release. Data were analyzed with GraphPad Prism software to obtain EC_{50} values or IC_{50} values, and the generalized Cheng-Prusoff (Leff and Dougall, 1993) equation was used to calculate pK_b values for antagonists.

Transfection of G Proteins. Expression vectors encoding $G\alpha q/i_5$ [a modified Gq replacing the five carboxyl-terminal amino acids of Gq with the corresponding residues from Gi (DCGLF); Coward et al., 1999], Gα16 (a promiscuous Gq family member), or null vector were transfected into HEK293 cells stably expressing $hH_3(445)$ using LipofectAMINE 2000 according to manufacturer's recommendations. Twenty-four hours after transfection, cells were dissociated with an enzyme-free cell dissociation buffer and seeded onto PEI-treated, black-walled, 96-well plates at a density of 75,000 to 100,000 cells/well in Dulbecco's modified Eagle's medium. Forty-eight hours after transfection, cells were tested for modulation of either intracellular calcium levels or cAMP levels.

Measurement of Intracellular Calcium Levels. Coupling of the $hH_3(445)$ receptor to $G\alpha q/i_5$ or Gα16 was determined by measuring agonist-evoked increases in intracellular calcium using Fluo-4-AM, a calcium-sensitive fluorescent dye. Confluent cells were loaded with 8 μM Fluo-4-AM in Dulbecco's phosphate-buffered saline at room temperature for 1 to 2 h. After extensive washing to remove unincorporated dye, assays for agonist activity were performed by exposing the cells to various histamine H_3 receptor ligands and measuring the relative fluorescence using the fluorescent imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA). Assays for antagonist activity were performed in a similar manner except that cells were incubated with 30 nM RAMH in addition to the varying concentrations of the ligand being tested. Reference wells received 10 μM (final concentration) of the calcium ionophore A-23187 to control for dye loading and cell density. Fluorescence values obtained just before addition of test compound were subtracted from fluorescence values at all time points. Peak response values were determined at each concentration of ligand and are expressed as a percentage of the response obtained with the full agonist RAMH, where the RAMH response was calculated using GraphPad Prism nonlinear regression fitting of the RAMH dose-response data within each transfection group ($G\alpha q/i_5$ and Gα16). Data were analyzed with GraphPad Prism software to obtain pEC_{50} values or pIC_{50} values. pEC_{50}, pIC_{50}, and efficacy data were statistically analyzed using a one-way ANOVA followed by Tukey's pairwise comparison post hoc tests.

Adenylate Cyclase Assay. C6 cells or HEK293 cells stably expressing $rH_3(445)$ or $hH_3(445)$ either alone or in combination with transiently expressed G protein were plated on 96-well plates coated with either collagen IV or PEI at 75,000 to 100,000 cells per well. Cells were assayed the next day for modulation of forskolin (FSK)-stimulated (final concentration, 10 μM) cAMP levels, essentially as described previously (Esbenshade et al., 2003). Briefly, cells were incubated for 20 min with 1 mM 3-isobutyl-1-methylxanthine, a cAMP phosphodiesterase inhibitor, followed by a 5-min incubation with agonist and a subsequent 10-min incubation with 10 μM FSK. For assays of antagonist activity, cells were incubated with antagonist for 2 min before the addition of the agonist (30 nM RAMH) and subsequently FSK. Cells were hydrolyzed by the addition of 1 N HCl, agitated for 10 min, and neutralized with 1 N NaOH. The quantity of cAMP was determined using either scintillation proximity assay or [^{125}I] Flashplate assays, according to manufacturer's instructions. The data presented were calculated after normalization for the amount of cAMP produced in control wells (basal response) and are expressed as a percentage of the FSK-stimulated cAMP response for that assay. Data were analyzed with GraphPad Prism software to obtain pEC_{50} values or pIC_{50} values. For the cotransfection experiments, pEC_{50}, pIC_{50}, and efficacy data were analyzed statistically using a one-way ANOVA followed by Tukey's pairwise comparison post hoc tests.

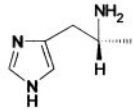
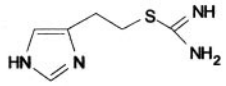
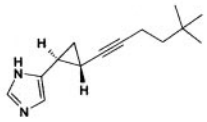
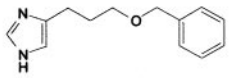
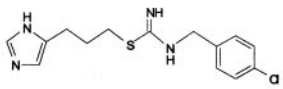
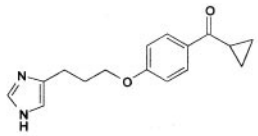
[^{35}S]GTPγS Binding Assay. Membranes from HEK293 or C6 cells expressing $hH_3(445)$ or from C6 cells expressing $rH_3(445)$ were prepared as described previously (Esbenshade et al., 2003). After dilution into GTPγS assay buffer (25 mM HEPES, 2.5 mM $MgCl_2$, and 75 mM NaCl, pH 7.4), approximately 10 μg of membrane protein was added to GTPγS assay buffer that contained 5.0 μM GDP, 0.5 nM [^{35}S]GTPγS, and the indicated concentrations of histamine H_3 ligands. After incubation at 37°C for 5 min, assays were terminated by the addition of cold buffer (50 mM Tris-HCl, 75 mM NaCl, and 2.5 mM $MgCl_2$, pH 7.6) and subsequent filtration onto a Packard Unifilter 96-well GF/B plate followed by extensive washing. The [^{35}S]GTPγS bound in each sample was determined by scintillation counting using a Topcount (PerkinElmer Life and Analytical Sciences) and was calculated as a percentage of that bound to control samples incubated in the absence of histamine H_3 ligand (basal). Data were analyzed with GraphPad Prism software to obtain pEC_{50} values.

Results

Histamine H_3 Receptor Binding Affinities. Displacement of [^{3}H]NAMH binding to membranes derived from rat or human cortex or to membranes prepared from C6 cells expressing either $hH_3(445)$ or $rH_3(445)$ was determined. As shown in Table 1, minimal differences in the average pK_i values within a species were found when membranes prepared from cortex or recombinantly expressing clonal cell lines were used. However, as described previously (for review, see Hancock et al., 2003), significant potency differences were found for some compounds when binding was compared across species (Table 1). Hence, the rank order of potencies for binding to $rH_3(445)$ (GT-2331 > imetit > clobenpropit > RAMH > ciproxifan > proxyfan) differs from that seen for binding to $hH_3(445)$ (imetit > clobenpropit > RAMH > GT-2331 > proxyfan > ciproxifan). Hill slopes, as reported in Table 1, approached unity regardless of the compound and receptor type assayed.

Modulation of Neurotransmitter Release. Clobenpropit, ciproxifan, GT-2331, and proxyfan all behaved as apparent competitive antagonists in the reversal of RAMH-mediated inhibition of contractility elicited in GPI tissue by electrical stimulation (Fig. 1A). Clobenpropit was most potent, with a pA_2 value of 10.35, followed by ciproxifan (pA_2 = 8.14), GT-2331 (pA_2 = 7.67), and proxyfan (pA_2 = 7.54).

TABLE 1
Competition binding affinities for the displacement of [^{3}H]*N*-α-methylhistamine binding to histamine H_3 receptors endogenously present in rat or human cortex (CTX) or stably expressed as the rat or human full-length form [rH_3(445), or hH_3(445), respectively] in C6 cells (for nomenclature, see Hancock et al., 2003)

		$pK_i \pm$ S.E.M. (n_H)			
		RAT CTX	rH_3(445)	HUMAN CTX	hH_3(445)
	RAMH	8.71 ± 0.08 (0.96)	9.35 ± 0.20 (0.94)	9.16 ± 0.07 (0.93)	9.16 ± 0.08 (0.89)
	Imetit	9.53 ± 0.09 (0.96)	10.11 ± 0.10[b] (0.95)	9.79 ± 0.07 (0.97)	9.59 ± 0.09 (1.00)
	GT-2331	9.71 ± 0.10[a] (1.00)	10.13 ± 0.11[b] (0.98)	8.30 ± 0.09 (1.09)	8.59 ± 0.06 (1.02)
	Proxyfan	8.51 ± 0.05 (0.89)	8.62 ± 0.10 (0.88)	8.38 ± 0.13 (0.81)	8.35 ± 0.06 (0.95)
	Clobenpropit	9.45 ± 0.08 (0.98)	9.82 ± 0.07 (1.08)	9.11 ± 0.11 (0.89)	9.47 ± 0.04 (1.01)
	Ciproxifan	9.22 ± 0.05[a] (0.89)	9.38 ± 0.07[b] (0.90)	7.05 ± 0.07 (1.06)	7.20 ± 0.04 (0.84)

Data are the average of at least three determinations and are expressed as the average pK_i with standard error of the mean. Average Hill slopes (n_H) are given in parentheses.
[a] Values significantly different compared with human cortex binding ($P < 0.05$).
[b] Values significantly different compared with hH_3(445) binding ($P < 0.05$).

When tested for their ability to modulate potassium-stimulated [^{3}H]histamine release from rat cerebral cortical synaptosomes (Fig. 1B), both clobenpropit and ciproxifan fully reversed the histamine-mediated inhibition of release, with pK_b values of 9.14 and 9.12, respectively. However, GT-2331 and proxyfan did not completely reverse the histamine-mediated inhibition of release (84 and 82%, respectively, of the percentage of maximum histamine release), but they did antagonize it, with resulting pK_b values of 8.13 and 8.22, respectively.

Given the incomplete antagonism observed above, GT-2331 and proxyfan were tested as agonists in either the EFS GPI assay (Fig. 2A) or the rat cerebral cortical synaptosomal neurotransmitter release assays (Fig. 2B). RAMH and imetit, two H_3 agonists, inhibited the EFS contractility in the GPI segments, with pEC_{50} values of 7.49 and 7.80, respectively, although imetit was less efficacious than RAMH (45 versus 25% of the maximum contractility elicited by EFS). Proxyfan showed a slight inhibition of the contractility (76% of the maximum), with a pEC_{50} value of 6.92, whereas little inhibition was observed with GT-2331 (Fig. 2A). RAMH and imetit were equally efficacious in their ability to inhibit potassium-stimulated [^{3}H]histamine release from rat cerebral cortical synaptosomes, with pEC_{50} values of 8.12 and 9.07, respectively (Fig. 2B). GT-2331 and proxyfan also inhibited [^{3}H]histamine release, with pEC_{50} values of 7.31 and 6.45, respectively, although the inhibition observed was much less than that produced by the full agonists RAMH and

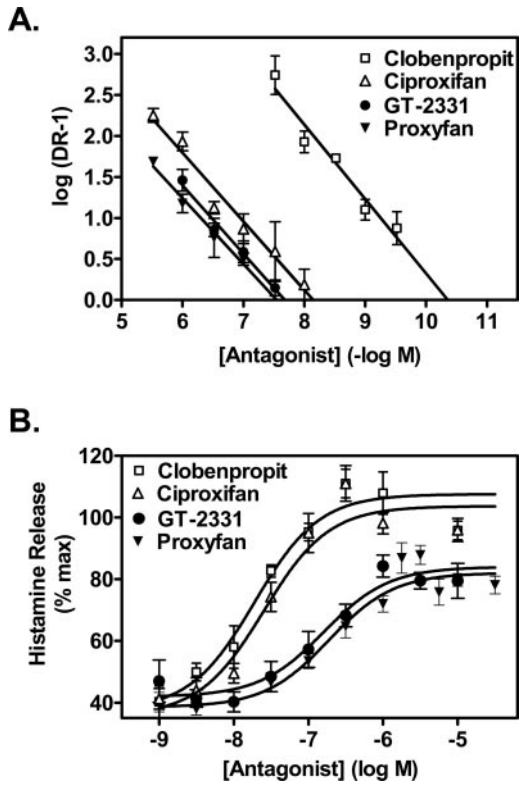


Fig. 1. Antagonism of RAMH- or histamine-mediated inhibition of neurotransmitter release. A, histamine H_3 receptor ligands were tested for reversal of RAMH-mediated inhibition of EFS GPI contractions, as described under *Materials and Methods*. Each data point shown is the average of at least three independent experiments performed in triplicate. Data were subjected to Schild analysis (Schild, 1947) using GraphPad Prism, and the resulting pA_2 values were determined. B, histamine H_3 receptor ligands were tested for reversal of 1 μM histamine-mediated inhibition of potassium-stimulated [^{3}H]histamine release from rat cerebral cortical synaptosomes, as described under *Materials and Methods*. Each data point shown is the average of at least three independent experiments performed in triplicate. Data are reported as the percentage of the maximum release of [^{3}H]histamine produced by incubation with 15 mM potassium. Data were analyzed using GraphPad Prism, the resulting IC_{50} values were determined, and pK_b values were calculated using the generalized Cheng-Prusoff equation (Leff and Dougall, 1993).

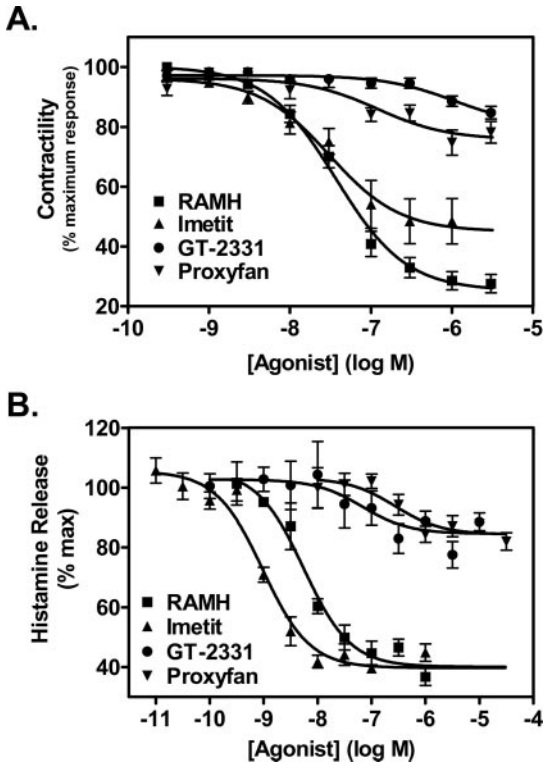


Fig. 2. Inhibition of neurotransmitter release. A, histamine H_3 receptor ligands were tested for inhibition of EFS GPI contractions, as described under *Materials and Methods*. Data are reported as the percentage of the maximum contractility produced by EFS. Each data point shown is the average of at least three independent experiments performed in triplicate. Data were analyzed using GraphPad Prism, and the resulting EC_{50} values were determined. B, histamine H_3 receptor ligands were compared for their ability to inhibit potassium-stimulated [^{3}H]histamine release from rat cerebral cortical synaptosomes, as described under *Materials and Methods*. Each data point shown is the average of at least three independent experiments performed in triplicate. Data are reported as the percentage of the maximum release of [^{3}H]histamine produced by incubation with 15 mM potassium. Data were analyzed using GraphPad Prism, and the resulting EC_{50} values were determined.

imetit (85% of the maximum histamine release compared with 40% for the full agonists). The weak partial agonism produced by GT-2331 and proxyfan was H_3 receptor-dependent, as evidenced by reversal of the inhibition of [^{3}H]histamine release with the specific H_3 antagonist clobenpropit (data not shown).

Inhibition of FSK-Stimulated cAMP Accumulation. Initial studies performed with C6 cells stably expressing either $hH_3(445)$ or $rH_3(445)$ did not reveal any antagonism by either GT-2331 or proxyfan of the RAMH-mediated inhibition of 10 μM FSK-stimulated cAMP accumulation (data not shown), whereas clobenpropit and ciproxifan fully reversed the inhibition caused by RAMH, with pK_b values of 9.03 and 9.10, respectively, in C6 cells expressing $rH_3(445)$, and 8.35 and 6.59, respectively, in C6 cells expressing $hH_3(445)$. Rather, GT-2331 and proxyfan alone were equally efficacious to RAMH and imetit in their ability to completely inhibit 10 μM FSK-stimulated cAMP accumulation in C6 cells expressing $rH_3(445)$ (Fig. 3A). Imetit was most potent in inhibiting cAMP accumulation in these cells, with an average pEC_{50} value of 9.66, followed in rank order by RAMH and GT-2331, which were essentially identical (8.67 and 8.72, respectively), and finally proxyfan (7.83) (Fig. 3A). Similar to the response in cells expressing the rat receptor, all four compounds were full agonists in mediating inhibition of FSK-stimulated cAMP accumulation in C6 cells expressing $hH_3(445)$ (Fig. 3B). However, the rank order of potencies was slightly different than with the rat form of the receptor. Imetit was again the most potent, with an average pEC_{50} value of 8.39, followed in rank order by RAMH (8.01), proxyfan (6.86) and GT-2331 (6.68) (Fig. 3B). The inhibition of FSK-stimulated cAMP levels was mediated by the H_3 receptor, since clobenpropit, a highly specific H_3 antagonist, reversed the inhibition produced by GT-2331 and proxyfan (data not shown).

Modulation of [^{35}S]GTPγS Binding. The ability of imetit, GT-2331, and proxyfan to modulate [^{35}S]GTPγS binding to membranes prepared from C6 cells expressing either $rH_3(445)$ or $hH_3(445)$ receptors was determined and was compared with the effects elicited by the full agonist RAMH (Fig. 4). Whereas imetit acted as a full agonist relative to RAMH for [^{35}S]GTPγS binding mediated by $rH_3(445)$, GT-2331 and proxyfan were only partial agonists, exhibiting less than 50% of the stimulation seen with RAMH or imetit (Fig. 4A). GT-2331 and imetit exhibited similar potencies of high affinity (pEC_{50} values of 8.60 and 8.69, respectively), whereas

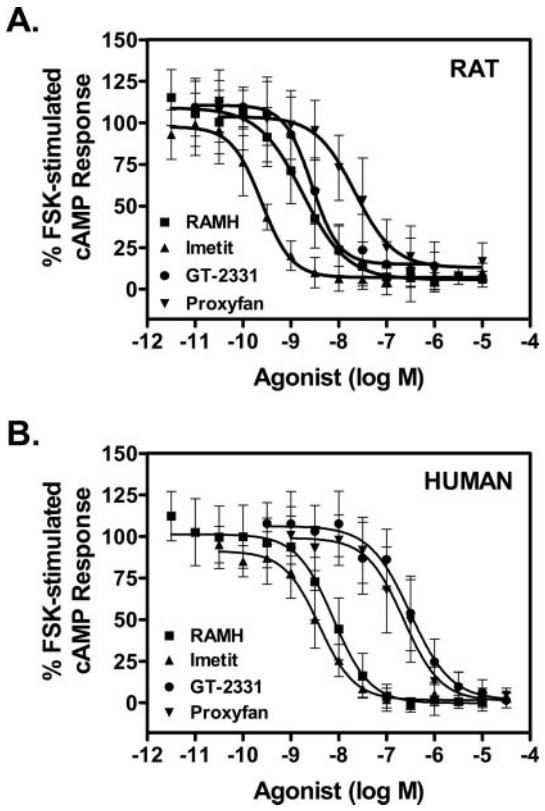


Fig. 3. Modulation of FSK-stimulated cAMP production in C6 cells expressing the rat or human histamine H_3 receptor. C6 cells expressing either $rH_3(445)$ (A) or $hH_3(445)$ (B) were incubated with 10 μM FSK in the presence of various concentrations of RAMH, imetit, GT-2331, or proxyfan, as described under *Materials and Methods*. The data are expressed as the percentage of the FSK-stimulated response observed and are the average of at least three independent experiments performed in triplicate. Data were analyzed using GraphPad Prism, and the resulting EC_{50} values were determined.

RAMH and proxyfan were less potent (pEC_{50} values of 7.65 and 7.33, respectively).

When assayed in membranes prepared from C6 cells expressing $hH_3(445)$ receptors, imetit was not as efficacious as RAMH, displaying approximately 86% of the [^{35}S]GTPγS binding stimulated by RAMH (Fig. 4B). GT-2331 and proxyfan behaved as partial agonists at the human isoform, stimulating approximately 61 to 65% of the [^{35}S]GTPγS binding seen upon incubation with RAMH, an effect that was slightly larger than that observed with the rat isoform. Imetit was the most potent compound in the C6 cells expressing $hH_3(445)$, with a pEC_{50} value of 8.53, followed by RAMH (8.02), and finally by GT-2331 and proxyfan, whose potencies (7.40 and 7.49, respectively) were essentially equivalent.

[^{35}S]GTPγS binding was examined in another cell line, HEK293 cells, stably expressing $hH_3(445)$. Imetit, GT-2331, and proxyfan, behaved as partial agonists in this cell line, stimulating approximately 75 to 80% of the [^{35}S]GTPγS binding seen upon incubation with RAMH (Fig. 4C). Imetit and RAMH displayed similar potencies (pEC_{50} values of 8.71 and 8.42, respectively), whereas GT-2331 and proxyfan were less potent, with pEC_{50} values of 7.50 and 7.68, respectively.

Effect of Receptor Density and the Type of G Protein on Modulation of Functional Responses by $hH_3(445)$. HEK293 cells stably expressing $hH_3(445)$ were transfected with DNA encoding either vector alone (Fig. 5), vector coding

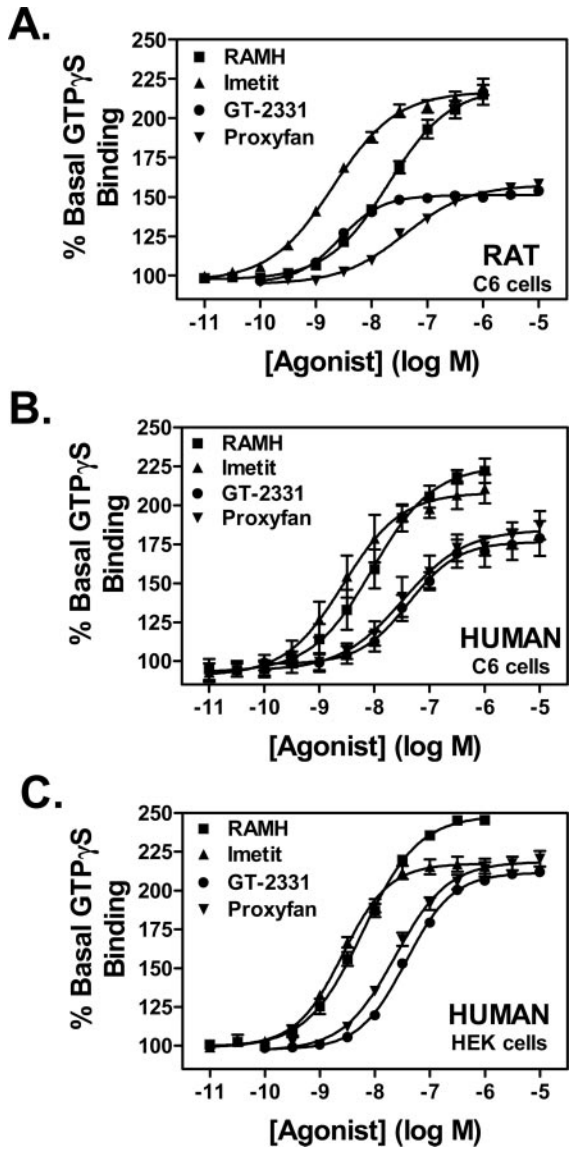


Fig. 4. Stimulation of [^{35}S]GTPγS binding. Membranes prepared from C6 cells expressing either $rH_3(445)$ (A) or $hH_3(445)$ (B) or from HEK cells expressing $hH_3(445)$ (C) were incubated at 37°C for 5 min with 0.5 nM [^{35}S]GTPγS and the indicated concentrations of histamine H_3 ligands, as described under *Materials and Methods*. The amount of [^{35}S]GTPγS bound was determined and is expressed as a percentage of the amount of [^{35}S]GTPγS bound under basal conditions (with buffer alone). Data were analyzed with GraphPad Prism software to obtain EC_{50} values.

for the promiscuous G protein α subunit Gα16 (Fig. 6), or vector coding for Gαq/i_5 (Fig. 7). Coexpression of the G protein subunits did not affect H_3 receptor density, as determined by saturation binding assays using [^{3}H]NAMH (data not shown). Cells transfected with vector alone showed no increases in intracellular calcium levels when incubated in the presence of RAMH, imetit, GT-2331, or proxyfan (Fig. 5A), whereas the inhibition of FSK-stimulated cAMP accumulation observed with each of these compounds was robust and equally efficacious (Fig. 5B). Although the majority of the studies with HEK293 cells expressing $hH_3(445)$ were performed with a line stably expressing the receptor at approximately 2.7 pmol/mg, adenylate cyclase assays were also performed on HEK293 cells stably expressing $hH_3(445)$ at low receptor densities (approximately 0.48 pmol/mg), Despite

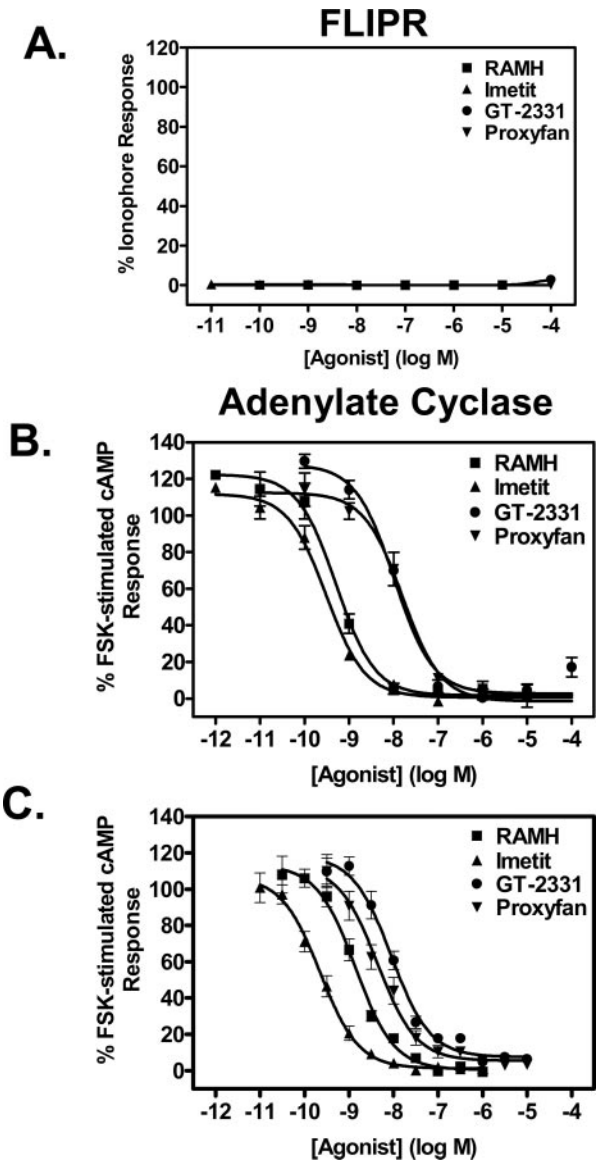


Fig. 5. Modulation of intracellular calcium levels and FSK-stimulated cAMP levels in HEK cells expressing $hH_3(445)$. In A and B, HEK293 cells stably expressing the $hH_3(445)$ were transiently transfected with null vector as a control for the experiments presented in Figs. 6 and 7. These cells were loaded with the calcium-sensitive fluorescent dye Fluo-4-AM and incubated with either A-23187, an ionophore, or the histamine H_3 ligands indicated in the figure legend (A). Fluorescence was measured using the FLIPR, with the intracellular calcium response calculated as described under *Materials and Methods*. The data are expressed as the percentage of the response elicited by the ionophore. These cells were also subjected to adenylate cyclase assays, as described under *Materials and Methods* (B). Adenylate cyclase assays were also performed on HEK293 cells stably expressing only 0.48 pmol/mg H_3 receptor (C) compared with approximately 2.7 pmol/mg in the HEK293 cells used for the other studies. The data are expressed as the percentage of the amount of cAMP produced upon incubation with FSK alone.

greatly reduced levels of $hH_3(445)$ expression, GT-2331, proxyfan, and imetit still showed equivalent efficacy as RAMH for the inhibition of FSK-stimulated cAMP levels (Fig. 5C).

HEK293 cells stably expressing $hH_3(445)$ and transiently expressing Gα16 were examined both for H_3 agonist-mediated increases in intracellular calcium levels (Fig. 6A) and for inhibition of FSK-stimulated cAMP levels (Fig. 6B). RAMH stimulated a dose-dependent increase in intracellular calcium in cells transfected with Gα16 (Fig. 6A). Imetit also stimulated intracellular calcium mobilization, however, to a much lower extent than that seen with RAMH. In contrast, stimulation of calcium mobilization by GT-2331 and proxyfan was minimal in these cells, but the response was dose-dependent (Fig. 6A). When the same cells were assayed for modulation of FSK-stimulated cAMP levels, RAMH, imetit, GT-2331, and proxyfan were all equally efficacious in the inhibition of cAMP accumulation (Fig. 6B). In Table 2, efficacies are reported, relative to the RAMH-mediated response, as are the potencies determined, expressed as pEC_{50} values. The rank order of the potencies determined in cells expressing Gα16 was imetit $\geq$ RAMH $\geq$ proxyfan $>$ GT-2331 when assayed for calcium mobilization, whereas the potency order for the cyclase assays was imetit $\geq$ RAMH $>$ GT-2331 $\geq$ proxyfan.

Assays for antagonism of RAMH-mediated increases in intracellular calcium levels in cells expressing $hH_3(445)$ and Gα16 revealed that clobenpropit and ciproxifan were fully efficacious, whereas GT-2331 and proxyfan inhibited approximately 50 to 60% of the RAMH-mediated response and imetit less than 20% (Fig. 6C; Table 3). The discrepancy between the efficacies of the compounds for stimulation of calcium mobilization compared with their efficacies for inhibition of the RAMH-mediated stimulation of calcium mobilization may reflect a nonequilibrium state that is common in this system due to the rapid calcium response that is detected by the FLIPR (Miller et al., 1999). In adenylate cyclase assays, neither GT-2331 nor proxyfan showed any antagonism of the RAMH-mediated inhibition of FSK-stimulated cAMP levels, whereas clobenpropit and ciproxifan fully attenuated the RAMH response (Fig. 6D; Table 3). This lack of an antagonist response by GT-2331 and proxyfan was consistent with the fully efficacious agonist response observed in the adenylate cyclase assays.

The pharmacology of these compounds was subsequently examined in the HEK293 cells stably expressing $hH_3(445)$ and transiently expressing Gαq/i_5 (Fig. 7). The maximal stimulation of intracellular calcium mobilization attained by imetit did not differ from that seen with RAMH (Fig. 7A). GT-2331 and proxyfan were partial agonists, eliciting approximately 50% of the maximal response observed with RAMH (Fig. 7A; Table 2). RAMH inhibited the FSK-stimulated cAMP response to a lesser degree (65% inhibition; Fig. 7B) than that seen in Gα16 (87% inhibition; Fig. 6B) or null vector (98% inhibition; Fig. 5B) cotransfected cells. However, imetit, GT-2331, and proxyfan still showed efficacy equivalent to the RAMH-mediated inhibition of cAMP accumulation observed (Fig. 7B; Table 2). Rank order of potencies for the calcium mobilization assays was RAMH $\geq$ imetit $>$ proxyfan $>$ GT-2331, whereas the order for inhibition of cAMP accumulation was RAMH = imetit $>$ GT-2331 $\geq$ proxyfan (Table 2).

Assays in cells expressing $hH_3(445)$ and Gαq/i_5 revealed that clobenpropit and ciproxifan fully antagonized the RAMH-mediated increases in intracellular calcium levels, whereas GT-2331 and proxyfan inhibited only approximately 35% of the response (Fig. 7C; Table 3). Imetit, consistent with demonstrating full agonist activity in this assay, showed no inhibition of the RAMH-mediated increases in intracellular calcium levels (Fig. 7C; Table 3). Whereas clobenpropit and ciproxifan attenuated RAMH-mediated inhibition of FSK-

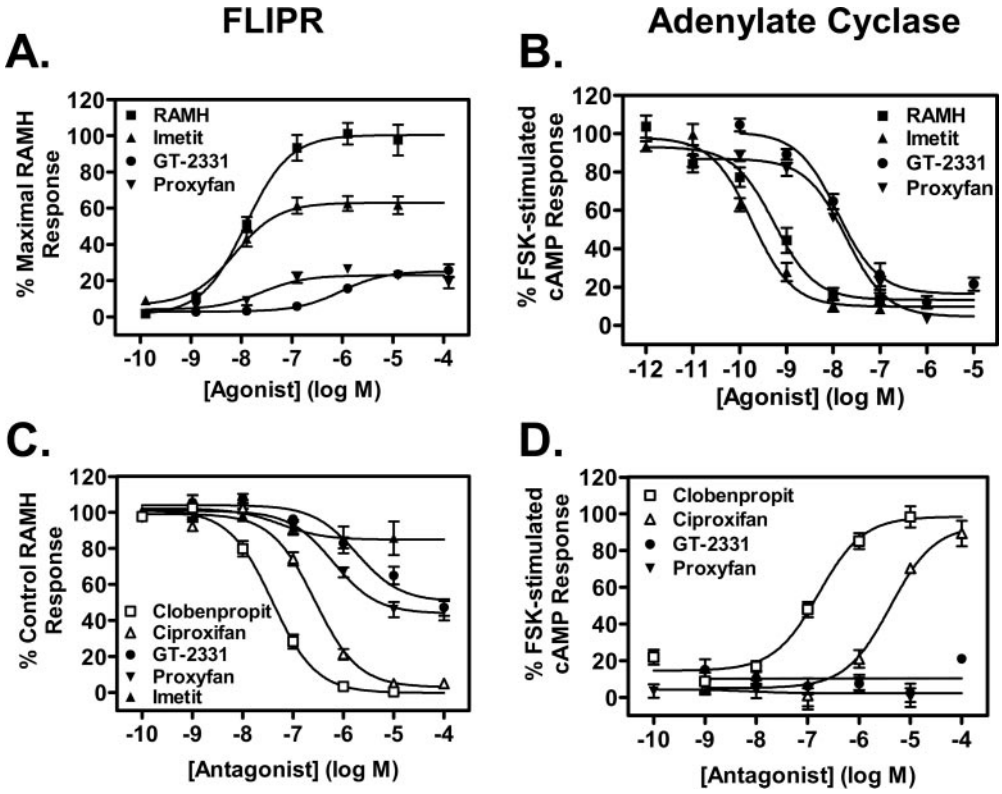


Fig. 6. Modulation of intracellular calcium levels and FSK-stimulated cAMP levels in HEK cells coexpressing $hH_3(445)$ and $G\alpha16$. Intracellular calcium levels (FLIPR assays) and cAMP levels (adenylate cyclase assays) were determined in HEK293 cells stably expressing $hH_3(445)$ and transiently expressing the α subunit of G16, a promiscuous Gq family member (see *Materials and Methods*). Histamine H_3 receptor ligands were tested for their ability to increase intracellular calcium levels alone (A) or to inhibit RAMH-mediated increases in intracellular calcium levels (C). The data are expressed as the percentage of the maximal response elicited by RAMH. In analogous experiments, the H_3 ligands were tested for their ability to either inhibit FSK-stimulated cAMP levels (B) or to reverse the inhibition of cAMP levels mediated by RAMH (D). Data were analyzed with GraphPad Prism software, and EC_{50} (A and B) or IC_{50} (C and D) values were calculated and are reported in Tables 2 and 3, respectively. The data shown are the average of three independent experiments performed in triplicate.

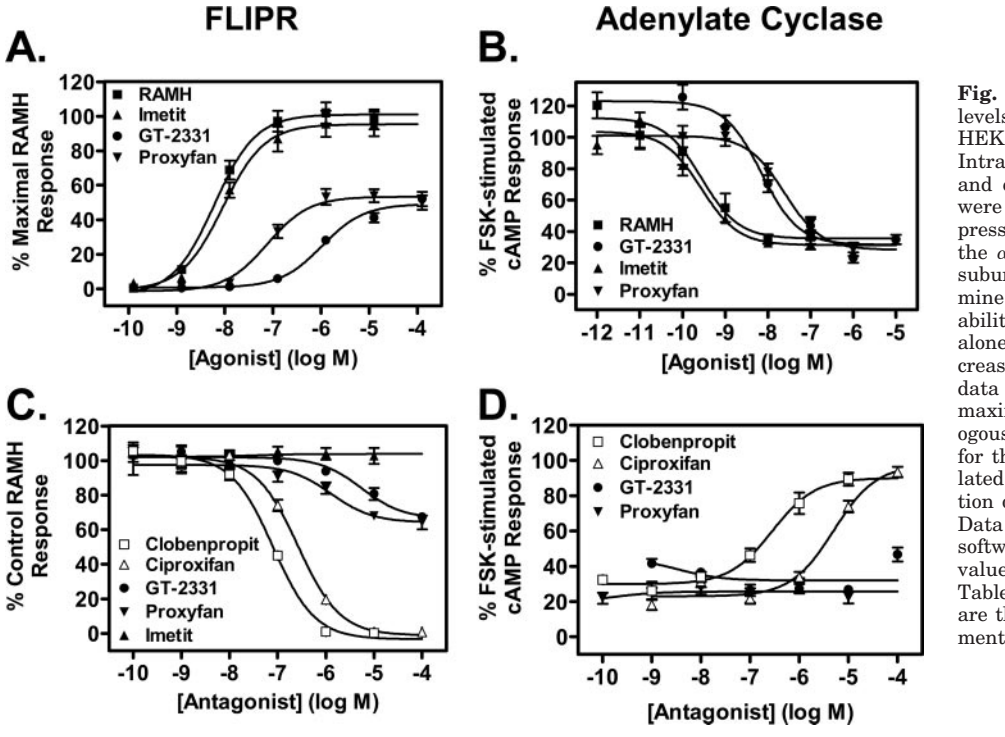


Fig. 7. Modulation of intracellular calcium levels and FSK-stimulated cAMP levels in HEK cells coexpressing $hH_3(445)$ and $G\alpha q/i_5$. Intracellular calcium levels (FLIPR assays) and cAMP levels (adenylate cyclase assays) were determined in HEK293 cells stably expressing $hH_3(445)$ and transiently expressing the α subunit of Gq/i_5, a chimeric G protein subunit (see *Materials and Methods*). Histamine H_3 receptor ligands were tested for their ability to increase intracellular calcium levels alone (A) or to inhibit RAMH-mediated increases in intracellular calcium levels (C). The data are expressed as the percentage of the maximal response elicited by RAMH. In analogous experiments, the H_3 ligands were tested for their ability to either inhibit FSK-stimulated cAMP levels (B) or to reverse the inhibition of cAMP levels mediated by RAMH (D). Data were analyzed with GraphPad Prism software, and EC_{50} (A and B) or IC_{50} (C and D) values were calculated and are reported in Tables 2 and 3, respectively. The data shown are the average of three independent experiments performed in triplicate.

stimulated cAMP accumulation, GT-2331 and proxyfan had no affect on the RAMH-mediated response (Fig. 7D; Table 3).

Discussion

Although the imidazole-containing histamine H_3 receptor ligands GT-2331 and proxyfan were originally classified as antagonists (Hüls et al., 1996; Tedford et al., 1998), subsequent reports revealed complex pharmacological effects, including neutral antagonism, partial to full agonism, and even protean agonism (Morisset et al., 2000; Rouleau et al., 2002; Wulff et al., 2002; Gbahou et al., 2003; Liedtke et al., 2003). Our studies were designed to further clarify this complex pharmacology and to gain insight into mechanisms of H_3-mediated signaling. To do this, we used both native tissues and recombinant expression systems to examine the differ-

TABLE 2
Histamine H_3 receptor ligand potencies and relative efficacies for calcium mobilization as detected using FLIPR or for inhibition of FSK-stimulated cAMP levels (cyclase)

	Gα Subunit Expressed	FLIPR		Cyclase	
		pEC_{50}	Relative Efficacy	pEC_{50}	Relative Efficacy
RAMH	G16	7.92^{b}	$1.00^{b,c,d}$	$9.26^{b,c}$	1.00
	Gq/i_5	$8.22^{b,c}$	$1.00^{b,c}$	$9.56^{b,c}$	1.00
GT-2331	G16	$6.03^{a,c,d}$	0.25^{a}	$7.91^{a,d}$	0.97
	Gq/i_5	$5.98^{a,c,d}$	$0.49^{a,d}$	$8.15^{a,d}$	1.06
Proxyfan	G16	7.61^{b}	$0.23^{a,d}$	$7.73^{a,d}$	1.10
	Gq/i_5	$7.11^{a,b,d}$	0.53^{a}	$7.61^{a,d}$	1.11
Imetit	G16	8.14^{b}	$0.63^{a,c}$	$9.73^{b,c}$	1.03
	Gq/i_5	$8.04^{b,c}$	0.95^{b}	$9.56^{b,c}$	1.06

Values significantly different from [a]RAMH, [b]GT-2331, [c]proxyfan, and [d]imetit within assay type and equivalent G protein ($P < 0.05$).

TABLE 3
Histamine H_3 receptor ligand potencies and relative efficacies for the inhibition of RAMH-mediated stimulation of calcium mobilization, as detected using FLIPR or for reversal of RAMH-mediated inhibition of FSK-stimulated cAMP levels (cyclase)
Due to minimal or no antagonist response, statistics were not calculated for imetit.

	G α Subunit Expressed	FLIPR		Cyclase	
		pIC_{50}	Relative Efficacy	pIC_{50}	Relative Efficacy
Clobenpropit	G16	$7.41^{b,c,d}$	$1.00^{c,d}$	6.79^{b}	1.00
	Gq/i_5	$7.10^{c,d}$	$1.00^{c,d}$	6.55^{b}	0.92
Ciproxifan	G16	$6.57^{a,c}$	$0.97^{c,d}$	5.39^{a}	0.96
	Gq/i_5	$6.58^{c,d}$	$1.00^{c,d}$	5.30^{a}	1.00
GT-2331	G16	$5.74^{a,b}$	$0.50^{a,b}$	N.D.	N.D.
	Gq/i_5	$5.26^{a,b,d}$	$0.34^{a,b}$	N.D.	N.D.
Proxyfan	G16	6.20^{a}	$0.56^{a,b}$	N.D.	N.D.
	Gq/i_5	$5.89^{a,b,c}$	$0.36^{a,b}$	N.D.	N.D.
Imetit	G16	6.94	0.19	—	—
	Gq/i_5	N.D.	N.D.	—	—

N.D., not detectable; —, not determined.
Values significantly different from [a]clobenpropit, [b]ciproxifan, [c]GT-2331, and [d]proxyfan within assay type and equivalent G protein ($P < 0.05$).

ential pharmacology displayed by these compounds. Furthermore, signaling through different G proteins was performed to examine the role that the G protein played in the observed pharmacology.

In studies of neurotransmitter release in isolated GPI tissue, both GT-2331 and proxyfan seemed to act as competitive inhibitors, similar to clobenpropit and ciproxifan (Fig. 1A), whereas incomplete antagonism was noted in the synaptosomal neurotransmitter release assay (Fig. 1B). Further examination showed that GT-2331 and proxyfan displayed weak partial agonism in both assays that measured modulation of neurotransmitter release (Fig. 2), and even imetit was not as efficacious as RAMH in the GPI assay. Contrary to these results, in adenylate cyclase assays performed in C6 cells expressing either $rH_3(445)$ or $hH_3(445)$, GT-2331 and proxyfan were full agonists, inhibiting the FSK-stimulated cAMP production to a level equivalent to imetit or RAMH (Fig. 3). Full agonism was also observed in HEK293 cells expressing $hH_3(445)$ at high or low receptor densities, indicating that the degree of agonism was independent of receptor density (Fig. 5).

Whereas coupling of H_3 receptors to adenylate cyclase occurs through Gi (Lovenberg et al., 1999), measurement of functional activity via [^{35}S]GTPγS binding is reflective of coupling that occurs to a broader pool of G proteins present in the cell. In C6 cells expressing $rH_3(445)$, GT-2331 and proxyfan stimulated less than half of the incorporation of [^{35}S]GTPγS produced upon incubation with RAMH or imetit (Fig. 4A). Although greater efficacy was observed in C6 or HEK293 cells expressing $hH_3(445)$, GT-2331 and proxyfan were still partial agonists. Unlike the full agonist response in cells expressing $rH_3(445)$, imetit was less efficacious than RAMH in stimulating [^{35}S]GTPγS binding to C6 or HEK293 cells expressing $hH_3(445)$. Hence, agonist efficacy differed depending on whether the cells were assayed for modulation of cAMP levels or for stimulation of [^{35}S]GTPγS binding. Increased efficacy in the cAMP assay could reflect amplification of signaling from the G protein to the effector, adenylate cyclase, resulting in saturation of a pathway component. However, since full agonism for cAMP assays is still observed in cells with low receptor density, the differences in efficacy may instead reflect differential ligand-bound receptor conformations that couple with varying efficiencies to different types of G proteins, as is discussed in more detail below.

To examine the role of the G protein in the differential pharmacology observed for these compounds, receptor coupling through different types of G protein was determined simultaneously in HEK293 cells coexpressing $hH_3(445)$ and Gα16. Whereas GT-2331, proxyfan, and imetit were equally efficacious agonists compared with RAMH in signaling through endogenous Gαi to inhibit FSK-stimulated cAMP production (Fig. 6B), these compounds behaved as partial agonists when assayed for their ability to signal through Gα16 to increase intracellular calcium levels (Fig. 6A). In fact, GT-2331 and proxyfan showed very weak partial agonist activity and were more effective at antagonizing the RAMH-mediated increase in intracellular calcium levels (Fig. 6C). Interestingly, coexpression of Gα16 in HEK293 cells resulted in decreased maximal responsiveness for agonist-mediated inhibition of FSK-stimulated cAMP accumulation by all the ligands tested (87% inhibition compared with 98% with the null vector transfectants), despite no change in $hH_3(445)$ expression levels.

Since GT-2331 and proxyfan behaved as full agonists when signaling through Gαi, whereas demonstrating antagonism when signaling through Gα16, a Gq family member, we decided to see what pharmacology was found in cells coexpressing a Gαq subunit containing the five carboxyl-terminal amino acids of Gi ($Gαq/i_5$). Whereas imetit was not as efficacious as RAMH when assayed for increasing intracellular calcium levels in cells coexpressing Gα16, it was fully efficacious in cells expressing $Gαq/i_5$. Both GT-2331 and proxyfan greatly increased (doubled) their efficacy in these cells, although they were still only 50% as efficacious as RAMH. Once again, GT-2331, proxyfan, and imetit were as efficacious as RAMH to inhibit FSK-stimulated cAMP production. However, the degree of maximal inhibition achieved by all of the compounds tested in the $Gαq/i_5$ cotransfected cells was only 65%, markedly less than that seen with Gα16 cotransfectants (87% inhibition) and greatly decreased compared with control cells (98% inhibition). Since expression levels of the H_3 receptor were not affected by cotransfection with G protein, the decreased responsiveness to the cyclase assay by the full agonist RAMH is probably the result of displacement of the endogenous Gαi with Gα16 or $Gαq/i_5$. Although this effectively meant that less H_3 receptor was functionally available to couple with endogenous Gαi, GT-2331, proxyfan, and imetit still displayed full agonist activity in cyclase as-

says. The differential efficacy of signaling observed in calcium assays for each compound when cells were cotransfected with equivalent amounts of DNA encoding either $G\alpha q/i_5$ or $G\alpha 16$ coupled with the differences observed in maximal inhibition of FSK-stimulated cyclase assays probably reflect increased coupling efficiency of the H_3 receptor with $G\alpha q/i_5$ versus $G\alpha 16$.

Many of the efficacy differences observed could result from varying coupling efficiencies with the different G proteins and signal transduction components across systems. However, the maintenance of full agonist activity by GT-2331, proxyfan, and imetit in cyclase assays performed at greatly reduced H_3 expression levels, as well as the observation that the maximal responsiveness for all compounds was reduced on coexpression with either $G\alpha q/i_5$ versus $G\alpha 16$, seem inconsistent with simple efficiency differences, since under these conditions, GT-2331 and proxyfan would be expected to show partial agonist activity rather than the full agonism observed.

In addition to the efficacy differences observed, assay-dependent changes in rank orders of potency were also noted. In adenylate cyclase assays using C6 cells expressing $rH_3(445)$, the potency order was imetit > RAMH ≥ GT-2331 > proxyfan, whereas that observed for [^{35}S]GTPγS binding was imetit > GT-2331 > RAMH > proxyfan. Also, in $G\alpha 16$ coexpressing cells, the potency order for calcium mobilization (imetit ≥ RAMH ≥ proxyfan > GT-2331) differed from the potency order for inhibition of FSK-mediated cAMP production (imetit ≥ RAMH > GT-2331 ≥ proxyfan). Similarly, in $G\alpha q/i_5$-coexpressing cells, the potency order for calcium mobilization (RAMH ≥ imetit > proxyfan > GT-2331) differed somewhat from the potency order for inhibition of FSK-mediated cAMP production (RAMH = imetit > GT-2331 ≥ proxyfan), where proxyfan was 13-fold more potent than GT-2331 in the calcium mobilization assays and 3-fold less potent in the cyclase assay, despite nearly equipotent binding affinities.

Pharmacological differences in receptor-mediated signaling pathways similar to those reported in the current studies may be indicative of the presence of alternative receptor conformations (Kenakin, 2002). Either clear differences in efficacy of ligands or relative potency reversals offer evidence for the presence of alternative receptor conformations (Kenakin, 2003). Thus, one possible explanation for the differences in efficacy and the potency reversals observed in the different H_3-mediated signaling systems by imetit, GT-2331, and proxyfan is the presence of different ligand-bound active state conformations that couple differentially to the signaling systems. This could occur by differential ligand-selection or ligand-induction of multiple active states that are capable of differential signaling (Kenakin, 2003; Kobilka, 2005). Consistent with our results, a previous study by Uveges et al. (2002) noted disparity in the pharmacological properties of imetit and impentamine when comparing cAMP effects in cells expressing the human H_3 receptor to measurements of intracellular calcium in cells expressing both the receptor and $G\alpha q/i_5$. Those authors suggested that the agonist-receptor complexes formed by these compounds were fundamentally different from that of other full agonists like histamine. The differential pharmacology demonstrated by GT-2331, proxyfan, and even imetit in the current studies, including the observed dependence on the G protein system used, may help to explain the range of previously reported ligand classifications for GT-2331 and proxyfan (Hüls et al., 1996; Tedford et al., 1998; Morisset et al., 2000; Rouleau et al., 2002; Wulff et al., 2002; Gbahou et al., 2003; Liedtke et al., 2003).

In addition to pharmacological observations that support differential conformations for GPCRs, mutagenesis studies and fluorescence lifetime spectroscopy have been used to demonstrate heterogeneous native and ligand-bound conformations. Using these techniques, a growing number of GPCRs have been shown to exist in a range of conformational states, including β_2-adrenergic receptors (Wenzel-Seifert and Seifert, 2000; Ghanouni et al., 2001), bombesin receptors (MacKinnon et al., 2001), adenosine A_1 receptors (Cordeaux et al., 2000), the calcitonin receptor (Watson et al., 2000), and others (for review, see Kenakin, 2003). Although our pharmacological data are suggestive of alternative conformations, it is not as direct or definitive as fluorescence lifetime spectroscopy. However, an advantage of pharmacological studies is that they can be done with native receptors in the membrane environment rather than a non-native receptor extracted from membranes.

In addition to signaling events, distinct ligand-bound conformations may differentially regulate other receptor processes, including receptor phosphorylation, desensitization, internalization, and oligomerization (Kenakin, 2002; Kenakin 2003). Future studies will be needed to ascertain whether these events occur for the histamine H_3 receptor and whether ligands differentially regulate them. The compounds used in the current studies that regulated G protein-mediated signaling differentially should be useful tools for such experiments.

Acknowledgments

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