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Generation and Characterization of Highly Constitutive Active Histamine H3 Receptors

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ABSTRACT

Constitutive activation of G-protein-coupled receptors is a well recognized phenomenon, and G-protein-coupled receptor antagonists have been found to possess inverse agonist activity. Constitutive activation of histamine H3 receptor is recently documented in in vivo as well as in recombinant receptor systems in vitro. Several H3 antagonists have been shown to act as inverse agonists and such profiles of H3 antagonists have been implicated in their pharmacological functions. Here we report the construction and characterization of a highly constitutive active H3 receptor (MT6), in which the 357 alanine residue was converted to lysine (A357K). We generated a series of mutated H3 receptors and their functions were examined in human embryonic kidney (HEK) 293 cells. Among them, induced mutation at the amino acid 357 position (A357K) showed a dramatically enhanced response to thioperamide-induced cAMP accumulation compared with the cells expressing wild-type (WT) H3 receptors, suggesting that the mutation rendered receptors to high constitutive activity. We further characterized by ligand binding assays using membrane fractions, and K_i values of imetit (agonist) and proxyfan (partial agonist) against the MT6 receptors were lower compared with those observed in WT H3 receptors. In contrast, H3 antagonists (thioperamide, ciproxifan, and GT2016) with inverse agonism displayed increased K_i values against the MT6 receptors (2.5- to 5.8-fold), demonstrating more a prominent effect of inverse agonists to the constitutive active receptor. Taken together, these data suggested that A357K mutation in the H3 receptor increased the population of active state receptors that preferably binds to agonists than inverse agonists, which could be termed as a constitutively active mutant of H3 receptor.

G-protein-coupled receptors (GPCRs) play a major role in signal transduction and are the targets of a large number of therapeutic drugs. Classical models of GPCRs require agonist occupation of receptors to activate signal transduction pathways. A widely accepted model used to describe agonist activation of GPCRs is the ternary complex model, which accounts for the cooperative interactions among receptor, G-protein, and agonist (De Lean et al., 1980). This model has recently been extended to accommodate the observations that several GPCRs can activate G-proteins in the absence of agonists, and that mutations in different structural domains of the GPCRs can enhance the agonist-independent (constitutive) activities. This led to the "extended ternary complex (ETC) model" (Samama et al., 1993). In this model, the receptor exists in an equilibrium between an inactive state (R) and an active state (R*) in the absence of drug. Binding of agonist to receptors stabilizes R* causing G-protein-coupling and activation of cellular responses whereas binding of inverse agonist stabilizes the R at the expense of R*. It has been reported that the primary structure and the expression level of receptors are major factors that govern the equilibrium and therefore determines the intrinsic basal activity of GPCRs. For wild-type receptors, R predominates and there is minimal receptor activity in the absence of agonist. In contrast, a high level of receptor expression or specific mutation in receptors increases the concentration of R*, leading to the increased activities in the absence of agonists and the enhanced susceptibility to inverse agonists.

In some disease states such as familial male precocious puberty (luteinizing hormone receptor) (Shenker et al., 1993), hyperfunctioning thyroid adenoma (thyrotropin receptor) (Parma et al., 1993), and retinitis pigmentosa (rhodopsin) (Rao et al., 1994), naturally occurring point mutations that lead to constitutive activation of GPCRs have been implicated as the cause of these disease phenotypes. Several investigators have demonstrated that amino acid substitutions in specific domains have produced constitutively active adrenergic receptors (Kjelsberg et al., 1992; Ren et al., 1993; Samama et al., 1993), muscarinic receptors (Spalding et al., 1995), dopamine D_5 receptors (Tiberi and Caron, 1994), and serotonin 5-hydroxytryptamine$_{2C}$ receptors (Herrick-Davis

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; ETC model, extended ternary complex model; NAMHA, *N*-α-methylhistamine; DMEM, Dulbecco's modified Eagle's medium; PTX, pertussis toxin; IBMX, 3-isobutyl-1-methylxanthine; HEK293 cell, human embryonic kidney 293 cell; ADR, adrenergic receptor; WT mH3, wild-type mouse H3; MT, mutated.

et al., 1997). In addition, many drugs classically thought as antagonists were subsequently found to have inverse agonist activities to their receptor (Chidiac et al., 1994).

The histamine H3 receptor was identified pharmacologically as a presynaptic autoreceptor regulating the release of histamine from histaminergic neurons (Arrang et al., 1983). Subsequently, human, guinea pig, rat, and mouse H3 receptors were cloned and shown to functionally couple to G_i proteins (Lovenberg et al., 1999, 2000; Tardivel-Lacombe et al., 2000; Chen et al., 2003). Recently, it is reported that the H3 receptor can signal without agonist stimulation in several recombinant receptor systems (Wieland et al., 2001; Wulff et al., 2002), as well as in vivo systems (Morisset et al., 2000), suggesting that the H3 receptor is one of the few GPCRs that could modulate physiological processes by means of their constitutive activities. In terms of clinical aspects, pharmacological agents with high inverse efficacy would be desirable if it were necessary to lower basal receptor activity and inverse agonists open the possibility for a new therapeutic strategy for the H3 receptor.

The H3 receptor is regarded as a therapeutic target for the central nervous system functions such as cognitive and memory processes, attention-deficit hyperactivity disorder, epilepsy, and sleep-wakefulness (Leurs et al., 1998). Recently, H3-disrupted mice demonstrated an obese phenotype that was characterized by increased body weight, food intake, adiposity, and reduced energy expenditure (Takahashi et al., 2002). It has been strongly suggested that H3 receptor regulates central nervous system-mediated physiological processes, either by control of histamine neurons itself, or by coordinating regulation of serotonin-, dopamine-, or noradrenaline-containing neurons (Schlicker et al., 1994).

In this study, we constructed and characterized a series of mutated H3 receptors and showed the highly constitutive activity of mutated H3 receptor (MT6) by demonstrating dramatic enhancement of the G_i signal, which is accompanied by the decreased binding affinities to inverse agonists. It is conceivable that utilization of this mutant H3 receptor might be a very useful tool to screen inverse agonists for the H3 receptor, as well as to elucidate the physiological importance of H3 receptor constitutive activities.

Materials and Methods

Materials. Thioperamide was obtained from Tocris Cookson (Ellisville, MO). All other H3 ligands were synthesized at Banyu Tsukuba Research Institute. Dulbecco's modified Eagle's medium (DMEM), Opti-MEM I, L-glutamine, penicillin, streptomycin, HEPES, LipofectAMINE, G418, and pertussis toxin (PTX) were purchased from Invitrogen (Carlsbad, CA). 3-Isobutyl-1-methylxanthine (IBMX) and histamine was obtained from Wako Pure Chemicals (Osaka, Japan). Forskolin, bovine serum albumin, and fetal bovine serum were purchased Sigma-Aldrich (St. Louis, MO). $[^3H]N$-α-methylhistamine (NAMHA) was obtained from PerkinElmer Life Sciences (Boston, MA; specific activity, 82 Ci/mmol).

Construction of Mutated Receptor cDNA and Expression Vectors. Wild-type mouse H3 (WT mH3) receptor cDNA was ligated into the mammalian expression vector pcDNA3.1 (Invitrogen). The mutated (MT) receptors were constructed from WT mH3 using the polymerase chain reaction. Mutagenic primers were designed complementary to amino acids 352–357 of the native mH3 cDNA, whereas changing amino acid 352 from Arg (CGG) to Lys (AAG), 353 from Asp (GAC) to Ala (GCC), 354 from Lys (AAG) to His (CAC), 357 from Ala (GCC) to Leu (CTC), Ile (ATC) or Lys (AAG). The mutated DNA fragments were sequenced by ABI Prism 377 DNA sequencer using BigDye terminator cycle sequencing kit (Applied Biosystems, Warrington, UK) to confirm the respective nucleotide sequences.

Cell Culture and Transfections. Human embryonic kidney (HEK) 293 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 2 mM L-glutamine at 37°C and 10% CO_2. Twenty-four hours before transfection, cells were seeded at 80% confluence in 100-mm dish. Cells were transfected with WT mH3 or MT receptors cDNA by LipofectAMINE. This was accomplished by combining 60 μl of LipofectAMINE with 10 μg of plasmid per 100-mm dish. Transfection was performed in Opti-MEM I (reduced-serum medium) for 6 h at 37°C. The cells were allowed to recover for 24 h before the addition of G418. Colonies that survived G418 selection (1 mg/ml) were grown and tested. The expression of WT mH3 and MT receptors was confirmed by Northern blot analysis.

Ligand Binding. Membranes from cells expressing WT mH3 and MT receptor were prepared as previously described (Takahashi et al., 2002). Membrane (0.2 mg of protein) was incubated for 60 min in 0.2 ml of 50 mM Tris-HCl at room temperature with 1.6 nM $[^3H]N$-α-methylhistamine (60,000 dpm per minute per assay) with or without cold drugs and then filtered over 0.3% polyethylenimine-presoaked Whatman GF/C filters (Whatman, Maidstone, UK). Nonspecific binding was defined as the activity counts in the presence of 10 μM NAMHA. Filters were washed four times with 1 ml of 50 mM of Tris-HCl. The radioactivity of the filters was determined by liquid scintillation counting. Saturation experiments were performed with increasing concentrations of $[^3H]$NAMHA. K_d and B_{max} values were determined using nonlinear regression analysis. Data analysis was performed using the GraphPad Prism data analysis software package and curve-fitting program (GraphPad Software, Inc., San Diego, CA). Protein was assayed with a bicinchoninic acid reagent (Pierce, Cheshire, UK).

Measurement of cAMP. HEK293 cells stably expressing either WT mH3 or MT receptors were plated on a 24-well plate (10^5 cells per well). After overnight cultivation, cells were incubated in fetal bovine serum-free DMEM with 0.2% bovine serum albumin for 15 min, further incubated with DMEM containing 0.5 mM IBMX (a phosphodiesterase inhibitor) for 15 min, and then treated with or without forskolin (10 μM) and histamine (or thioperamide) for 15 min. The cells were treated with lysis buffer, and then 5 μl of the lysate was tested for measurement of cAMP concentration. cAMP accumulation levels were determined using the cyclic AMP enzyme immunoassay system (Amersham Biosciences, Inc., Piscataway, NJ). For experiments assessing the effects of PTX, cells were treated for 18 h before the cAMP accumulation assay with 100 ng/ml PTX in DMEM, supplemented as described above.

Results

Histamine H3 receptors have a putative seven membrane-spanning domain which is conserved in most of the G-protein-coupled receptors (Fig. 1a). Published studies of adrenergic receptors have indicated that coupling to specific G-proteins seemed to be predominantly mediated by the third intracellular loop, especially those regions in close proximity to the lumen of the plasma membrane. The carboxyl terminus of internal 3 (i3) loop of H3 receptor has a stretch of eight amino acids that are highly similar to the corresponding sequence of human m1 muscarinic receptors, α_{1B}-adrenergic receptors, and thyroid-stimulating hormone receptors (Fig. 1b). Among these eight amino acids, seven amino acids are identical between mouse and human H3 receptors, suggesting the functional importance of these conserved residues. Also, this region is reported to play critical roles in obtaining constitutive activities in native or mutated G-pro-

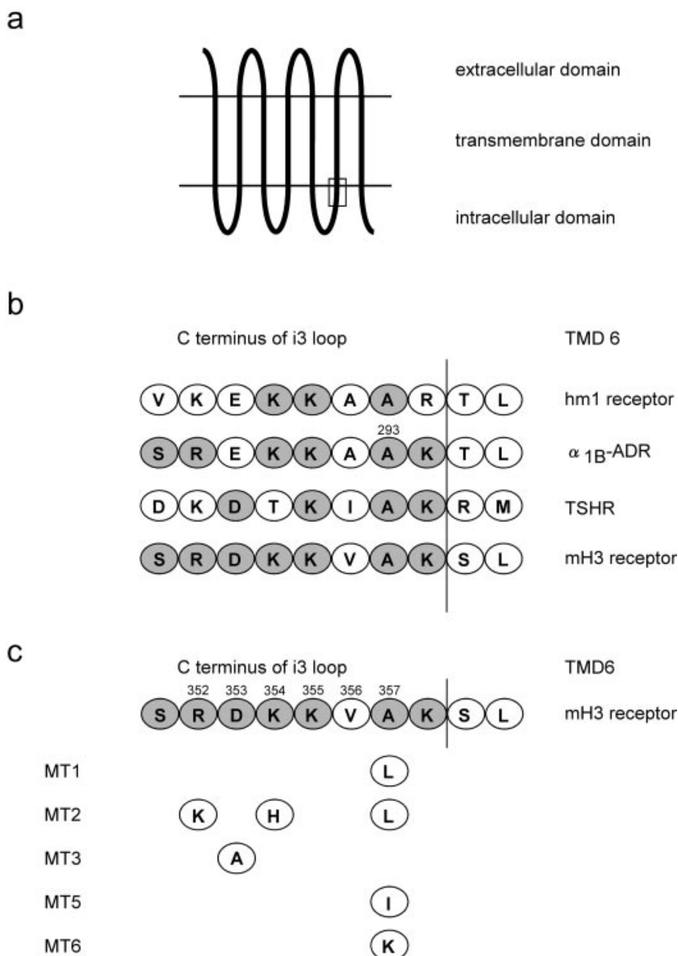


Fig. 1. Construction of mutated mouse H3 receptors. a, putative seven-transmembrane segment topography of the H3 receptor. Open square indicate border between 6th transmembrane domain and internal 3rd loop. b, comparison of C-terminal sequence in the i3 loop in human m1 muscarinic receptor, α_{1B}-ADR, thyroid-stimulating hormone receptor (TSHR), and mH3 receptor. Shaded amino acid residues indicate conserved amino acid between mouse H3 receptor and other GPCRs. c, schematic representation of five MT H3 receptors. Numbers indicate the amino acid count from the initiation Met.

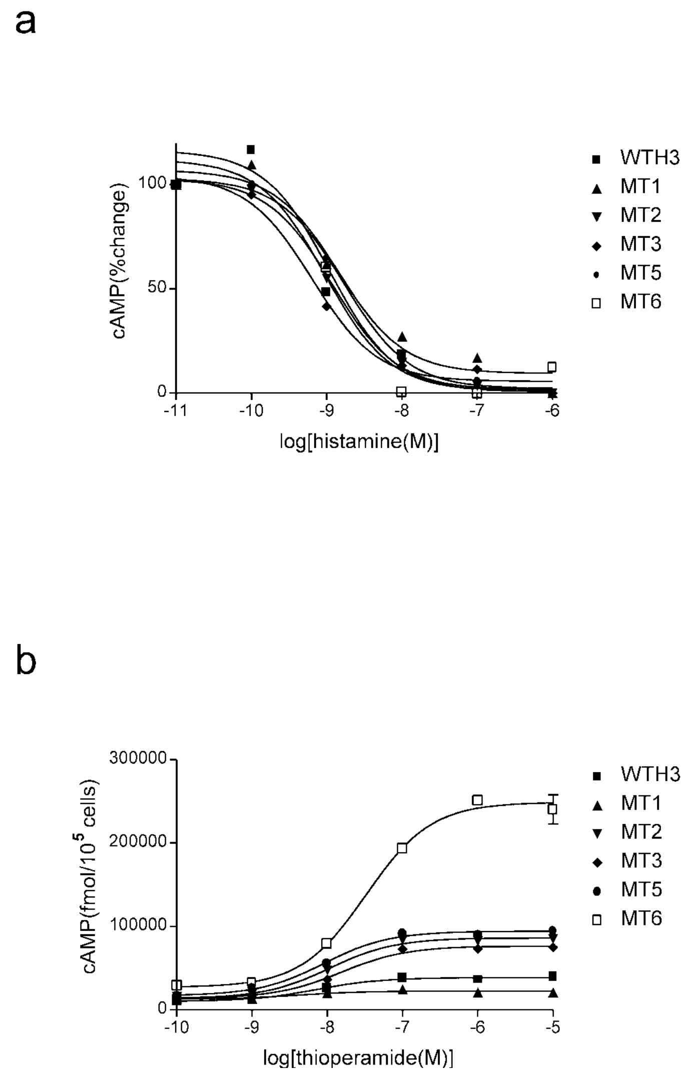


Fig. 2. Histamine and thioperamide response of WT H3 and MT receptors expressing HEK293 cells. a, effects of histamine on forskolin-evoked cAMP accumulation in HEK293 cells expressing WT H3 or MT receptors. b, effects of thioperamide on forskolin-evoked cAMP accumulation in HEK293 cells expressing WT H3 and MT receptors. Basal inhibition of forskolin stimulation in MT receptors was 1.01 for MT1, 1.33 for MT2, 1.28 for MT3, 1.84 for MT5, and 3.08 for MT6 (cAMP concentration, -fold difference to WT receptor). Symbols represent means with S.E.M. of the three independent experiments. HEK293 cells expressing WT H3 and MT receptors were stimulated with 10 μM forskolin in the presence of 0.5 mM IBMX and increasing concentrations of either histamine (a) or thioperamide (b) for 15 min at 37°C.

tein-coupled receptors (Kjelsberg et al., 1992; Parma et al., 1993; Claeysen et al., 1999). In an attempt to construct highly constitutive active H3 receptors, we have mutated several key residues in this region by point mutagenesis and created five different H3 receptor variants as shown in Fig. 1c (MT1: A357L, MT2: R352K, K354H, A357L, MT3: D353A, MT5: A357I, MT6: A357K).

We assessed the activities of WT H3 or H3 variants (MT1, 2, 3, 5, and 6) receptors in HEK293 cells by stable expression. To evaluate biochemical properties of these variant receptors, we first confirmed the activation of variant H3 receptors upon exposure to a native ligand, histamine. As expected, histamine inhibited forskolin-induced cAMP accumulations in cells expressing all five mutated clones and WT H3 receptors (Fig. 2a). We also observed no changes in the EC_{50} values of histamine, which are around 1.0 nM for WT, 1.4 nM for MT1, 1.2 nM for MT2, 0.6 nM for MT3, 1.7 nM for MT5, and 1.1 nM for MT6. These values are in agreement with other published studies (Wulff et al., 2002) for cAMP response to histamine.

To examine the constitutive activities of these mutated receptors, we decided to test the effect of thioperamide, an inverse agonist of the H3 receptor, on the G_i activity (Fig. 2b). Thioperamide was originally identified as an H3 receptor antagonist (Arrang et al., 1987) and has been used as a pharmacological agent to modulate H3 activities in vitro (Lovenberg et al., 1999) and in vivo (Hew et al., 1990). After the molecular identification of the H3 receptor gene, several groups have further confirmed the inverse agonist activity of thioperamide in in vitro and in vivo systems (Morisset et al., 2000; Wieland et al., 2001; Wulff et al., 2002). Consistent with these observations, thioperamide increased forskolin-induced cAMP production in HEK293 cells expressing WT H3 receptor in a concentration-dependent manner (Fig. 2b; WT), whereas thioperamide had no effect on the forskolin response in the parental cell line transfected with a plasmid

vector. In contrast, cells expressing MT6 (A357K) showed a dramatically enhanced response to thioperamide-induced cAMP accumulation compared with the cells expressing WT receptors. When compared at maximum responses to thioperamide (10^{-6} M), the MT6 variant caused a 6.9-fold increase in cAMP accumulation compared with WT H3 receptor. Increased cAMP productions by thioperamide were also observed in MT2 (R352K, K354H, A357L), MT3 (D353A), and MT5 (A357I) variants, showing intermediate activation between WT and MT6 receptors (2.3-fold for MT2, 2.0-fold for MT3, and 2.5-fold for MT5 at 10^{-6} M to WT H3 receptor). To our surprise, MT1 (A357L)-expressing cells did not show any change in cAMP levels in response to thioperamide, suggesting the 357 alanine is a critical amino acid for maintaining the constitutive activity of mouse H3 receptors.

Since H3 receptors are known to interact with G_i subtype of G-protein to modulate intracellular cAMP levels, constitutive activities of these mutant H3 receptors should be suppressed by PTX if these responses were mediated through specific interaction with appropriate G-protein i.e., G_i proteins. Pretreatment with PTX attenuated the cAMP accumulation in the cells expressing WT H3 receptor as expected (Fig. 3). Furthermore, thioperamide-induced increase in the forskolin-stimulated cAMP formation was completely terminated in cells expressing MT6 variant H3 receptors. These findings confirmed that the increased constitutive activities of MT6 receptors resulted from the augmentation of the intrinsic coupling with G_i protein, not G_s protein.

In ETC models, the constitutive activity of GPCRs is determined by an equilibrium between the population of active receptors (R*) and inactive receptors (R). Agonists shift an equilibrium to the state where R* predominantly exist, resulting in the enhanced signal transductions, whereas inverse agonists increase inactive receptor R at the expense of R*, thus resulting in decreased receptor activities in the presence or absence of agonists. Therefore, ideal constitutively active receptors will have increased affinity to agonists while showing decreased affinity to inverse agonists. To confirm this hypothesis, we performed a ligand binding assay using membrane fractions from WT or MT6 H3 receptors and a radioactive H3 agonist, [^{3}H]N-α-methylhistamine. One representative experimental result is shown in Fig. 4. In both WT and MT6 receptors, [^{3}H]N-α-methylhistamine bound specifically to one single binding site as determined by using nonlinear regression analysis, and the nonspecific binding of [^{3}H]N-α-methylhistamine was always <2.0% of total binding; specific binding to nontransfected HEK293 cells could not be detected (data not shown). The K_d values of [^{3}H]N-α-methylhistamine determined for the WT H3 or MT6 receptors were 2.19 and 2.04 nM, respectively, indicating similar affinity to the agonist between WT and constitutively active MT6 H3 receptors. Since the B_{max} value of WT was 2.07 pmol/mg of protein and that of MT6 receptors was 0.46 pmol/mg of protein, indicating that WT H3 receptors are more abundantly expressed in our cell lines. These observations have excluded the possibility that the enhanced constitutive activity of MT6 was due to the higher expression levels of the receptors.

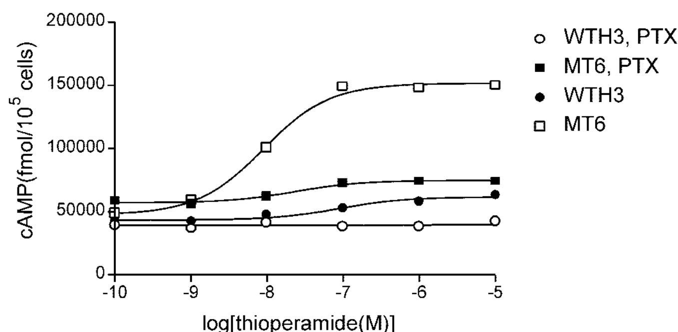
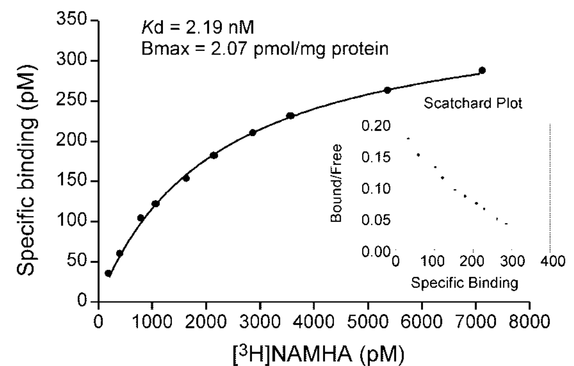


Fig. 3. PTX sensitivity of thioperamide induced cAMP changes in WT H3 and MT6 receptors expressed in HEK293 cells. Whole cell cAMP assays were performed on cells for 15 min at 37°C in the presence of 0.5 mM IBMX and 10 μM forskolin. Cells pretreated with or without 100 ng/ml PTX were incubated with indicated concentrations of thioperamide.

a



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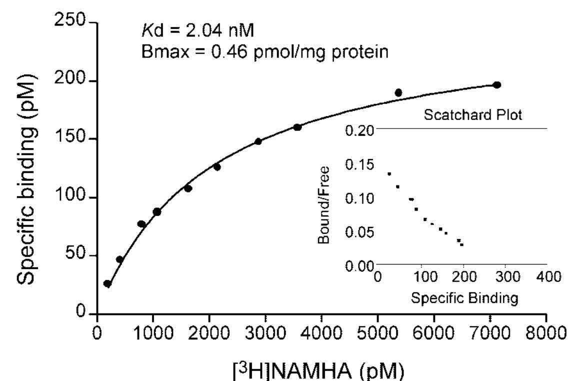

Fig. 4. Saturation analyses of [^{3}H]N-α-methylhistamine binding to membranes of WT H3 (a) and MT6 (b) receptors expressed in HEK293 cells. Saturation binding to WT H3 or MT6 membranes was performed with increasing concentration of [^{3}H]N-α-methylhistamine for 60 min at room temperature. Nonspecific binding, defined in the presence of 10 μM NAMHA and represented less than 2% of the total binding, was subtracted. The K_d values determined at the WT H3 or MT6 receptor expressed in HEK293 cells were 2.19 and 2.04 nM, and the B_{max} values were 2.07 and 0.46 pmol/mg of protein, respectively.

We also determined K_i values of WT and MT6 receptors to several H3 compounds in displacement studies using [^{3}H]*N*-α-methylhistamine and membranes from WT H3 or MT6 receptors. One representative dose-response curve (Fig. 5, a and b) and K_i values determined from these experiments were shown (Table 1). In agonists binding studies using imetit and proxyfan, the K_i value of these ligands were slightly reduced in MT6 receptors than in WT H3 receptor-expressing cell membranes. As Morisset et al. (2000) previously reported that the pharmacological profile of proxyfan might depend on the assay systems, they reported that proxyfan could act as a partial inverse agonist (arachidonic acid release) as well as partial agonist (cAMP decrease) in Chinese hamster ovary cells with high H3 receptor expression levels. In our cAMP assay, proxyfan caused a partial decrease in cAMP production in WT H3-expressing HEK293 cells, therefore we classified proxyfan as a partial agonist in our assay systems (data not shown). Furthermore, the H3 antagonists (thioperamide, ciproxifan, GT2016, and UCL2171) observed more prominent differences in affinities (K_i values) between the WT and MT6 receptors (Fig. 5, a and b; Table 1). As shown in Table 1, all the inverse agonists we tested had reduced K_i values, suggesting lower affinities against MT6 receptors than WT receptors. Among them, a decrease in affinity was most apparent with GT2016 (5.8-fold to WT receptors).

Discussion

We have constructed highly constitutively active H3 receptors (MT6; A357K) by induced point mutagenesis in the C-terminal portion of the third intracellular loop of mouse H3 receptors. This substitution resulted in three major biochemical modifications of the H3 receptors: 1) increased constitutive activity leading to agonist-independent inhibition of adenylyl cyclase in cells; 2) moderately increased affinity for some agonists; and 3) reduced affinities for antagonist acting as inverse agonists.

Several mutagenesis studies indicated that the C-terminal portion of the i3 might act in concert with other structural domains to determine the selectivity and efficiency of G-protein-coupling receptor. For the adrenergic receptors, it has been shown that the C-terminal portion of the i3 plays a crucial role in G-protein-coupling receptor (O'Dowd et al., 1988; Cotecchia et al., 1990; Liggett et al., 1991). Ren et al. (1993) previously showed that the T373K mutation adjacent to TM6 causes dramatically enhanced basal activation of G_i in the α_{2A}-ADR (Ren et al., 1993). In particular, α_{1B}-ADR showed that any amino acid substitution for Ala293 (Fig. 1b) could result in induction of constitutive activity (Kjelsberg et al., 1992). Our observations in H3 receptors are in agreement with these studies in that substitution of the Ala357 corresponding to Ala293 in α_{1B}-ADR, resulted in increased constitutive activity. It is interesting to note that substitution of Ala357 to Leu357 (MT1) resulted in loss of intrinsic constitutive activity of the receptors. Nevertheless, we were able to overcome this inactivation by concomitant mutation at adjacent residues (R352K and K354H; MT2). These findings might indicate that specific mutations in i3 loop result in the changes in receptor conformation that could lead to the modification of intrinsic activity of H3 receptors and also signified the importance of the Ala357 residue of H3 receptor in maintaining the activated state of receptors. Responsiveness to PTX in the constitutive activity of the MT6 confirmed that increased constitutive activity was caused by an enhanced coupling to intrinsic G_i pathway but not by promiscuous interaction with the other G-proteins such as G_s.

The two-state extended ternary complex model has been proposed to explain constitutive activation and inverse agonism, and this model predict that agonists/inverse agonists will have distinct affinities for an inactive state R and an active state R* of the receptor. Altered affinities of agonists and inverse agonists in constitutively active mutated recep-

TABLE 1

Affinities of several H3 receptor ligands to WT H3 and MT6 receptors

K_i values were calculated from IC_{50} values determined by competition binding for 1.6 nM [^{3}H]*N*-α-methylhistamine (Fig. 5). Membranes of HEK293 cells expressing WT H3 or MT6 receptor were incubated for 60 min at room temperature in the presence of increasing concentrations of the indicated drugs. Relative affinities are the MT6 K_i divided by the WT K_i for each drug.

Ligand	WT H3	MT6 (A357K)	Relative Affinity MT6/WT H3
Imetit	0.8	0.6	0.75
Proxyfan	8.3	5.3	0.64
Thioperamide	34.8	87.2	2.5
Ciproxifan	4.9	13.3	2.7
GT-2016	58.1	336.3	5.8
UCL2171	137.8	773.8	5.6

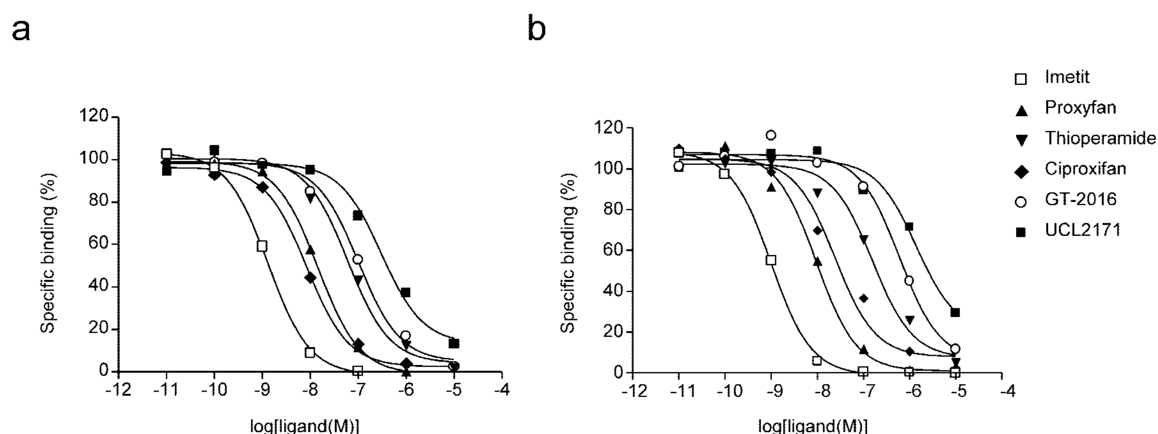

Fig. 5. Binding of H3 agonists and antagonists to the WT H3 (a) and MT6 (b) receptors. Competition binding for 1.6 nM [^{3}H]*N*-α-methylhistamine was performed for 60 min at room temperature with WT H3 or MT6 membranes. For agonists, imetit and proxyfan were used. Thioperamide, ciproxifan, GT2016, and UCL2171 were antagonists.

tors have been reported experimentally (Costa and Herz, 1989; Samama et al., 1994; Wurch et al., 1999; Wade et al., 2001). Our binding studies using several H3 receptor agonists and antagonists certainly corroborated with this model. Binding studies with MT6 variant of H3 receptor showed a slight increase in agonist affinities and a dramatic decrease in inverse agonist binding affinities compared with the WT H3 receptor. According to the published studies using α_{2A}-adrenergic receptors, observed alterations in agonist and inverse agonist affinities to the mutated receptors largely depended on the spontaneous activity of the receptors determined by the equilibrium between R and R* states (Wurch et al., 1999; Wade et al., 2001). Since the binding affinities of MT6 receptors to inverse agonists significantly differ from those of WT H3 receptors, the induced mutation of A357K might shift the equilibrium toward R* state dramatically. In several in vitro experiments, the constitutive activity of GPCRs expressed in cells was often attributed to a dramatically increased expression of the receptors because no alternation of the basal activities are observed in the cells expressing low to moderate levels of the same receptors. In our studies, the expression level of MT6 variants is less than that of WT receptors (0.46 pmol/mg of protein versus 2.07 pmol/mg of protein), suggesting that the increased constitutive activities observed in the MT6-expressing cells could not be simply explained by increased number of the expressed receptor. In addition, expression levels of MT5 (medium level constitutive activity, 0.78 pmol/mg of protein) and MT1 (no constitutive activity, 1.49 pmol/mg of protein) were also comparable to MT6 and WT, respectively, providing us further confirmation on receptor activation due to the changes in their structures. Taken together, presumed increased population of R* state might explain, at least partially, the highly constitutive activity of the receptors evidenced by the enhanced responsiveness to thioperamide.

In conclusion, we successfully generated a highly constitutive active H3 receptor by induced mutation of A357K (MT6) and demonstrated enhanced activation of G_i protein signaling and the increased affinities against the H3 ligands with remarkable inverse agonism, which could be termed as a constitutively active mutant of H3 receptor. This receptor, together with constitutively inactive receptor (MT1), might be a useful tool for the screening of inverse agonists as well as for the creation of transgenic mice, which have variable degrees of basal H3 receptor activities. In light of the foreseen therapeutic application of H3 antagonists, it remains to be established whether inverse agonists or neutral antagonists will be favored for clinical application. Therefore, molecular biological tools such as these mutant receptors may hold the key to further evaluation of therapeutic potential of H3 receptor-modulating compounds.

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