Colchicine: A Novel Positive Allosteric Modulator of the Human 5-Hydroxytryptamine_{3A} Receptor

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ABSTRACT

The actions of colchicine were examined with the two-electrode voltage-clamp technique and radioligand binding assays in mouse and human 5-hydroxytryptamine_{3A} receptors (5-HT_{3A}Rs) expressed in *Xenopus laevis* oocytes. Colchicine inhibited 5-hydroxytryptamine (5-HT)-evoked currents in oocytes expressing mouse 5-HT_{3A}Rs, with an IC₅₀ of 59.5 ± 3 μM. In contrast to the mouse receptor, coapplication of colchicine with 5-HT (<1 μM) strongly enhanced 5-HT-evoked currents in oocytes expressing human 5-HT_{3A}Rs. Colchicine applied alone did not induce a detectable current. In the presence of 0.5 μM 5-HT, the potentiation was concentration-dependent and reached the maximum (~100%) when 750 μM colchicine was applied. However, colchicine-dependent inhibition can be observed at 5-HT concentrations > 1 μM. In oocyte membranes expressing

mouse or human receptors, binding studies with colchicine (25 nM–1 mM) revealed no displacement of 1-methyl-*N*-((1*R*,3*r*,5*S*)-9-methyl-9 azabicyclo [3.3.1]nonan-3-yl)-1*H*-indazole-3 carboxamide ([³H]BRL-43694), suggesting that actions of colchicine do not occur at the ligand binding domain. Functional effects of colchicine on both receptors occurred in the absence of preincubation and after cold temperature incubation, suggesting that the microtubule-depolymerizing effects of colchicine play no role in modulation of receptor function. Studies with interspecies chimeric receptors demonstrated that the distal one third of the N terminus is responsible for the bidirectional modulation by colchicine. Collectively, these results suggest that colchicine modulates receptor function through loops C and/or F through a gating mechanism.

The 5-hydroxytryptamine₃ receptor (5-HT₃R) belongs to the superfamily of ligand-gated ion channels (LGICs) (Maricq et al., 1991) and shares a number of structural and functional homologies with other members, such as the nicotinic acetylcholine receptor (nAChR), GABA receptor (GABA_AR), and glycine receptor (GlyR) (Grenningloh et al., 1987; Schofield et al., 1987). To date, five 5-HT₃ receptor subunits have been cloned (A–E) (Maricq et al., 1991; Belelli et al., 1995; Niesler et al., 2007). Receptors composed of A-homers and A/B-heteromers have been shown to have functional significance in the central and peripheral nervous systems (for review, see Thompson and Lummis, 2006), and a recent study reported expression of functional A/C-, A/D-, and A/E-heteromers (Niesler et al., 2007). The

ligand binding domain (LBD) is located in the N terminus at the interface between subunits and consists of six loops, A to F. Loops A to C form the principal component of the LBD, and loops D to F form the complementary component of the LBD (for review, see Thompson and Lummis, 2006). Homology models using the acetylcholine-binding protein and biochemical studies have identified important residues for ligand binding on both the primary (loops A–C) and the complementary (loops D–F) interfaces of the binding site (Schreiter et al., 2003; Thompson et al., 2005).

The homomeric 5-HT_{3A}R is a good model system for exploring the ligand recognition sites of LGICs. Significant homology exists between the 5-HT_{3A}Rs among the six cloned species (80–95%). It is interesting that these small differences in amino acid composition are responsible for striking interspecies variation of responses to numerous drugs that act at the 5-HT recognition site, including curare (Miyake et al., 1995; Zhang et al., 2007), 2-methyl 5-HT (Maricq et al.,

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ABBREVIATIONS: 5-HT_{3A}R, 5-hydroxytryptamine_{3A} receptor; LGIC, ligand-gated ion channel; nAChR, nicotinic acetylcholine receptor; GABA_AR, GABA receptor; GlyR, glycine receptor; LBD, ligand binding domain; 5-HT, 5-hydroxytryptamine; BZD, benzodiazepine; MBS, modified Barth's solution; HWT, human wild-type receptor; MWT, mouse wild-type receptor; HWT A, MWT A; MWT A, MWT A; VDB, vesicle dialysis buffer; MDL-72222, 3-tropanyl-3, 5-dichlorobenzoate; TM, transmembrane; [³H]BRL-436, 1-methyl-*N*-((1*R*,3*r*,5*S*)-9-methyl-9 azabicyclo [3.3.1] nonan-3-yl)-1*H*-indazole-3 carboxamide; 2-OHMA, 3-2-hydroxy, 4-methoxybenzylidene anabaseine; PNU-120596, 1-(5-chloro-2, 4-dimethoxy-phenyl)-3-(5-methyl-isoxanol-3-yl)-urea.

1991; Belelli et al., 1995), 1-phenylbiguanide (Maricq et al., 1991), and benzylidene analogs of anabasine (Zhang et al., 2006). Loops C and F have been implicated in interspecies differential potency of curare and *m*-chlorophenylbiguanidine (Hope et al., 1999; Mochizuki et al., 1999; Zhang et al., 2007) and agonist function of 2-OHMBa (Zhang et al., 2006). The differential sensitivity of these drugs among interspecies 5-HT_{3A}Rs can be used to localize ligand binding domain(s) or gating residues through the use of interspecies chimeras and point mutations.

An important feature of all LGICs is the allosteric regulation of receptor function by various compounds. For example, PNU-120596 potentiates (Bertrand et al., 2008), whereas steroids either potentiate or inhibit the function of neuronal nAChRs (Paradiso et al., 2001). More allosteric modulators have been found for GABA_ARs than for any other LGIC. Such ligands include barbiturates, picrotoxin, progesterone metabolites, and benzodiazepines (BZDs) and non-BZDs, which act at the BZD recognition site (for review, see Sigel and Buhr, 1997; Da Settimo et al., 2007). Tropeines allosterically modulate the activity of GlyRs (Maksay et al., 2008).

The 5-HT_{3A}R is allosterically modulated by numerous drugs. Most of these drugs fall in the class of alcohols and anesthetics and, therefore, are not selective for the 5-HT_{3A}R. Examples of drugs that potentiate 5-HT_{3A}R-mediated currents include trichloroethanol (Lovinger et al., 2000) and volatile anesthetics (Machu and Harris, 1994). However, to date, 5-hydroxyindole and its analogs (van Hooft et al., 1997) are the only reported selective allosteric modulators of 5-HT_{3A}R function, albeit with weak potentiating actions effective only in the millimolar concentration range.

Colchicine is a microtubule-depolymerizing agent that competitively antagonizes GABA_ARs (Bueno and Leidenheimer, 1998) and GlyRs (Machu, 1998). In the present study, we determined the actions of colchicine on 5-HT_{3A}Rs. We found that colchicine inhibits the mouse 5-HT_{3A}R with a similar potency to that observed in the GABA_AR and α 2 GlyR (Bueno and Leidenheimer, 1998; Machu, 1998). Radioligand binding assays show colchicine does not displace [³H]BRL-43694, a competitive antagonist that binds at the 5-HT_{3A}R agonist recognition domain. More importantly, we found that colchicine potentiates the function of the human 5-HT_{3A}R at low 5-HT concentrations and antagonizes the receptor at higher 5-HT concentrations. By constructing interspecies chimeras, we identified 63 amino acid fragments within the distal one third of the N termini of the receptors that confer colchicine's potentiating actions.

Materials and Methods

Isolation of *Xenopus laevis* Oocytes. *X. laevis* frogs were kept in tanks of dechlorinated tap water on a 12-h light/dark cycle at 19°C and fed a diet of dehydrated liver in *X. laevis* I chow three times per week. Frogs were anesthetized by immersion in cold 0.12% 3-aminobenzoic acid ethyl ester for 20 min. After removal through a small surgical incision in the frog's abdomen, ovarian lobes were placed in modified Barth's Solution (MBS) containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, and 0.91 mM CaCl₂, pH 7.5.

Ovarian lobes were manually dissected into clumps of four to 10 oocytes and were then subjected to chemical separation and defolliculation. Clumps of oocytes were placed in medium containing 2 mg/ml collagenase type 2 and 83 mM NaCl, 2 mM KCl, 1 mM MgCl₂,

and 10 mM HEPES, pH 7.5, and gently rocked for 2 h. Oocytes were then removed to fresh collagenase medium and rocked gently for an additional 2 h. Lastly, oocytes were rinsed with MBS and stored in incubation media composed of ND96, containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.5, plus 10 mg/l streptomycin, 50 mg/l gentamicin, 10,000 units/l penicillin, 96 mg/l sulfamethoxazole, 19 mg/l trimethoprim, 0.5 mM theophylline, and 2 mM sodium pyruvate.

Construction of Chimeric Receptors. Mouse and human 5-HT_{3A}R cDNAs, provided by Drs. D. Julius and A. Miyake, respectively, were subcloned into pCR-Script Amp SK(+) (Stratagene, La Jolla, CA). Three unique restriction enzyme cleavage sites, NruI, SpeI, and NarI, were introduced in both mouse and human 5-HT_{3A}R cDNAs by site-directed mutagenesis (U.S.E. mutagenesis kit; GE Healthcare, Chalfont St. Giles, UK) of the nucleotides encoding the conserved residues, Thr181 and Arg244 in the mouse receptor cDNA and Thr176 and Arg239 in the human receptor cDNA, respectively. Numbering of the amino acids in the two receptors began with the initiating methionine. Primers were used to introduce SpeI and NarI, and selection pressure for the mutagenesis was performed as described previously by Zhang et al. (2006). Chimeric cDNAs were confirmed by dideoxynucleotide sequencing at the Biotechnology Core Facility at Texas Tech University (Lubbock, TX).

Transcription of cDNA to cRNA. The wild-type and chimeric cDNAs were linearized with BamHI, extracted with phenol-chloroform, precipitated with sodium acetate and ethanol, and resuspended in diethyl pyrocarbonate-treated water. The cDNAs were then transcribed with T3 mMESSAGE mMACHINE (Ambion, Austin, TX).

Microinjection of Oocytes with 5-HT₃ Receptor cRNAs. An aliquot of cRNA was centrifuged at 15,000g, and the ethanol was removed with a tuberculin syringe. After air-drying, the pellet was resuspended in a volume of diethyl pyrocarbonate water to yield a concentration of 5 to 30 ng of cRNA/50 nl. The cRNA was drawn up into a micropipette (10–20- μ m tip size). Fifty nanoliters of cRNA was injected into the animal/vegetal pole equator of each oocyte. Oocytes were stored in incubation medium in Corning cell well plates (Corning Inc., Corning, NY) at room temperature. Incubation medium was changed daily. Oocytes were recorded from days 2 through 7 after injection.

Electrophysiological Recordings. Oocytes were perfused in a 100- μ l volume chamber with MBS via a roller pump (Cole-Parmer Instrument Co., Vernon Hills, IL). Oocytes were impaled with two glass electrodes (1.2-mm outer diameter and 1–10-M Ω resistance) filled with 3 M KCl. Oocytes were voltage-clamped to –70 mV with a Warner Instruments Model OC-725C oocyte clamp (Hamden, CT). Clamping currents were plotted on a strip chart recorder (Cole-Parmer Instrument Co.). Serotonin, in the absence or presence of colchicine, was dissolved in MBS and applied to the oocytes for 30 s. For the cold temperature experiments, oocytes were incubated at least 2 h before recording and were continuously perfused with ice-cold MBS during recording.

Membrane Purification. Oocytes were injected with 100 ng of human wild-type receptor (HWT) or mouse wild-type receptor (MWT) cRNA and tested for expression using two-voltage-clamp electrophysiology on day 3. Oocytes were homogenized with a hand-held glass potter (~50 *X. laevis* oocytes) in ice-cold vesicle dialysis buffer (VDB) containing 96 mM NaCl, 10 mM MOPS, 0.1 mM EDTA, and 0.02% NaN₃, pH 7.5, 0.1 ml/oocyte + 10 μ l of protease inhibitor cocktail set III (Calbiochem, San Diego, CA). The homogenate was centrifuged for 10 min at 800g. The supernatant was collected, and the pellet was resuspended in VDB (0.1 ml/oocyte) and recentrifuged at 800g for 10 min. Supernatants were then combined and centrifuged for 1 h at 38,800g. The membrane pellet was resuspended in VDB (2–8 ml depending on expression) and divided into several batches for storage at –80°C. Protein content was measured with the Lowry assay (Lowry et al., 1951).

Radioligand Binding Assays. For K_d determinations, membrane homogenate was incubated at 37°C for 2 h with [³H]BRL-

43694 (0.1 nM–23 μ M). Specific binding was calculated as $X \times B_{\max}/(K_d + X)$, where X is radioligand concentration, $B_{\max}$ is maximal specific binding per milligram of protein, and K_d is the dissociation constant ($-1/\text{slope}$). For K_i determinations, aliquots of membrane were incubated at 37°C for 2 h with colchicine (25 nM–1 mM) and [3 H]BRL-43694 at the K_d of MWT (3.5 nM) and HWT (2.6 nM), respectively. Nonspecific binding was measured with MDL-72222, a 5-HT_{3A} receptor competitive antagonist.

Data Analysis. GraphPad Prism (GraphPad Software Inc., San Diego, CA) was used to calculate K_d , K_i , $B_{\max}$, EC_{50} and IC_{50} values, and Hill coefficients. GraphPad InStat was used to perform Student's t tests, one-way analysis of variance, Student's t test, and post hoc tests.

Results

Colchicine Inhibits 5-HT-Evoked Currents in *X. laevis* Oocytes Expressing Mouse 5-HT_{3A}Rs. Figure 1A illustrates typical responses of the mouse 5-HT_{3A}R to colchicine. In the absence of preincubation, colchicine clearly inhibited 5-HT-mediated responses in oocytes expressing the mouse 5-HT_{3A}R. Colchicine quickly washed out as a baseline response was obtained with the next application of 5-HT alone. A colchicine concentration-response curve was generated in oocytes expressing the mouse 5-HT_{3A}R (Fig. 1B). When coapplied with 0.5 μ M 5-HT, which generates 10% of the maximal current (EC_{10}), colchicine inhibited 5-HT-mediated currents in a concentration-dependent manner at concentrations ranging from 3 to 300 μ M. The colchicine concentration that reduced 5-HT-mediated currents by 50% (IC_{50}) was 59.5 ± 3 μ M. To examine whether microtubule depolymerization was involved in the colchicine-inhibitory action at the mouse 5-HT_{3A}R, the temperature sensitivity of the colchicine effect was measured (Table 1). For at least 2 h before recording, oocytes were incubated in ice-cold MBS, which completely depolymerizes oocyte microtubules (Gard, 1991). Colchicine at 50 and 200 μ M inhibited 5-HT-mediated currents by 43 and 82% in oocytes incubated at 4°C

for 2 h (Table 1), which is virtually identical to that observed in oocytes maintained at room temperature (43 and 80%, respectively).

Antagonism by Colchicine at Mouse 5-HT_{3A}Rs. To determine the mechanism(s) through which colchicine inhibits mouse 5-HT_{3A}Rs, we performed additional electrophysiological experiments and radioligand binding experiments. In the first set of experiments, we recorded 5-HT (0.25–500 μ M) concentration-response curves in the presence of increasing concentrations of colchicine, 200, 1000, and 10,000 μ M (Fig. 2). In the presence of increasing concentrations of competitive antagonists, 5-HT concentration-response curves shift rightward, with increases in 5-HT EC_{50} values; maximal current amplitudes are achieved in the presence of sufficient concentrations of 5-HT. In contrast, noncompetitive antagonists reduce the maximal response to 5-HT, even at very high 5-HT concentrations, and cause no shift in the 5-HT EC_{50} values. In Fig. 2A, the 5-HT concentration-response curves were shifted to the right as concentrations of colchicine increased. However, the inhibitory effect of colchicine (1000 and 10,000 μ M) was not completely overcome as 5-HT concentrations were increased. Therefore, it is not possible to determine from these experiments the mechanism(s) through which colchicine inhibits receptor function. The EC_{50} of 5-HT was 0.93 ± 0.04 μ M in the absence of colchicine. In the presence of colchicine (200, 1000, and 10,000 μ M), the EC_{50} values for 5-HT were 3.7 ± 0.3 , 13.4 ± 2.4 , and 81.4 ± 3.3 μ M, respectively.

To determine whether colchicine has any competitive action at the ligand binding domain for 5-HT, we performed radioligand binding assays (Fig. 2; Table 2). In Fig. 2B, saturation binding experiments were performed with the 5-HT_{3A}R competitive antagonist, [3 H]BRL-43694; a K_d of 3.53 ± 0.3 nM was obtained. In Fig. 2C, colchicine (25 nM–1 mM) did not displace [3 H]BRL-43694 (3.5 nM). These results

Fig. 1. Antagonist potency of colchicine at the mouse 5-HT_{3A}R. A, representative tracings of currents produced in a single oocyte expressing mouse 5-HT_{3A}Rs. Serotonin (0.5 μ M) was applied for 30 s to obtain a baseline current. Serotonin (0.5 μ M) was applied in the absence or presence of colchicine (300 μ M) for 30 s. An inhibition of ~92% of the 0.5 μ M 5-HT-induced baseline current was observed for the receptor. Washout of colchicine inhibition was observed with the next application of 5-HT 5 min later. B, concentration-response curve for colchicine was generated in oocytes expressing mouse 5-HT_{3A}Rs ($n = 4-7$). Colchicine (1–300 μ M) was coapplied with 0.5 μ M 5-HT for 30 s. An IC_{50} of 59.5 ± 3 μ M was obtained. The Hill coefficient was 1.3 ± 0.08 .

TABLE 1
Effect of temperature on colchicine modulatory actions on human and mouse 5-HT_{3A}Rs

5-HT _{3A} R	MWT				HWT			
	50 μ M Colchicine		200 μ M Colchicine		300 μ M Colchicine		1000 μ M Colchicine	
Temperature	22°C	4°C/2 h	22°C	4°C/2 h	22°C	4°C/2 h	22°C	4°C/2 h
Change (%)	42.9 ± 5.0	43.4 ± 3.0	80.4 ± 6.0	81.5 ± 2.5	79.3 ± 8.7	90.3 ± 4.3	-39.0 ± 7.9	-34.3 ± 5.0

Fig. 2. Colchicine antagonizes mouse 5-HT_{3A}Rs. **A**, concentration-response curves for colchicine were generated in oocytes expressing the mouse 5-HT_{3A}R ($n = 3-5$). Serotonin concentration-response curves were generated in the presence of colchicine (0, 200, 1000, and 10,000 μ M). The EC_{50} s generated for each of the curves were 0.93 ± 0.04 , 3.7 ± 0.3 , 13.4 ± 2.4 , and 81.4 ± 3.3 μ M, respectively. **B**, [³H]BRL-43694 binding to MWTA 5-HT_{3A} receptor-rich membranes. Membranes were incubated for 2 h with increasing concentrations of [³H]BRL-43694 (0.1–23 nM). Total ($\blacktriangledown$), specific ($\blacksquare$), and nonspecific ($\blacktriangle$) binding. Nonspecific binding was measured with MDL-7222, and specific binding was calculated by subtracting nonspecific from total binding. Scatchard plot of the binding data is shown. The K_d (3.5 ± 0.3 nM) was calculated as the negative reciprocal of the slope of three separate experiments. **C**, colchicine displacement of [³H]BRL-43694 binding to MWTA 5-HT_{3A} receptor-rich membranes. Membrane was incubated (2 h) with increasing concentrations of colchicine (25 nM–1 mM) and [³H]BRL-43694 at $K_d = 3.5$ nM. Total ($\blacktriangledown$), specific ($\blacksquare$), and nonspecific ($\blacktriangle$) binding. Nonspecific binding was measured with MDL-7222, and specific binding was calculated by subtracting nonspecific from total binding. No displacement of the radioligand was observed.

suggest that colchicine does not act at the 5-HT recognition site.

Allosteric modulation of ligand-gated ion channels produces shifts in agonist concentration-response curves, result-

TABLE 2

Colchicine effects on 5-HT potency at both MWTA and HWTA receptors

5-HT _{3A} Construct	MWTA	HWTA
[³ H]BRL-43694 K_d (nM)	3.53 ± 0.3	2.58 ± 0.6
Colchicine K_i (nM)	N.A.	N.A.
5-HT K_i (nM)	311 ± 0.5	1530 ± 230
5-HT + 300 μ M colchicine K_i (nM)	406 ± 0.37^a	738 ± 0.64^c
5-HT + 1000 μ M colchicine K_i (nM)	1250 ± 150^b	2940 ± 0.15^d

N.A., not applicable.

^a $p < 0.05$ Compared with 5-HT K_i for MWTA.

^b $p < 0.01$ Compared with 5-HT K_i for MWTA.

^c $p < 0.05$ Compared with 5-HT K_i for HWTA, Student's t test.

^d $p < 0.01$ Compared with 5-HT K_i for HWTA, Student's t test.

ing in altered agonist EC_{50} values in electrophysiological studies and in binding studies (Sigel and Buhr, 1997). For example, benzodiazepine agonists such as diazepam produce shifts in the GABA or GABA agonist, muscimol, concentration-response curves of the GABA_A receptor to the left, and benzodiazepine inverse agonists produce rightward shifts of the GABA or muscimol concentration-response curves. Benzodiazepines produce these changes by altering the affinity of GABA and other GABA agonists at their recognition site. To determine whether colchicine alters the affinity of 5-HT, we measured the displacement of [³H]BRL-43694 by 5-HT (0.25 nM–500 μ M) in the absence and presence of colchicine (300 and 1000 μ M) (Table 2). A similar approach has been used to examine PNU-69176E's allosteric modulation of 5-HT displacement of [³H]mesulergine at the 5-HT recognition site of the 5-HT_{2C} receptor (Im et al., 2003). We reasoned that if colchicine decreases the affinity of 5-HT at the 5-HT recognition site in the mouse receptor, then the potency of 5-HT in displacing [³H]BRL-43694 would be reduced. In the absence of colchicine, the 5-HT K_i was 311 ± 0.5 nM. Colchicine (300 and 1000 μ M) significantly increased the K_i values of 5-HT to 406 ± 0.37 and 1250 ± 150 nM, respectively (Table 2). These results suggest that colchicine acts at an allosteric site to modulate mouse 5-HT_{3A} receptor function.

Effects of Colchicine on 5-HT-Evoked Currents in *X. laevis* Oocytes Expressing Human 5-HT_{3A}Rs. In contrast with the mouse receptor, colchicine (300 μ M) enhanced currents evoked by 5-HT (0.5 μ M), an $\sim EC_5$ (Fig. 3A) in the human 5-HT_{3A} receptor. The EC_{50} for 5-HT is 1.39 ± 0.06 μ M. Stimulation occurred in the absence of preincubation. Colchicine quickly washed out because a baseline response was observed with the next application of 5-HT (0.5 μ M). To further explore the potentiating effects of colchicine on human 5-HT_{3A}R function, a colchicine (10–1000 μ M) concentration-response curve was generated in the presence of 0.5 μ M 5-HT (Fig. 3B). Colchicine enhanced 5-HT-evoked currents at concentrations as low as 25 μ M, and the potentiation reached the maximum when the colchicine concentration was increased to 750 μ M ($\sim 100\%$). A colchicine EC_{50} of 226.6 ± 25.1 μ M was obtained. Colchicine (200 μ M or 10 mM) in the absence of 5-HT produced no detectable current in oocytes expressing the human 5-HT_{3A}R (data not shown). Therefore, colchicine enhances human receptor function at a site other than the 5-HT recognition site.

Potentiating Effect of Colchicine at the Human 5-HT_{3A}R is 5-HT Concentration-Dependent and Microtubule-Independent. Figure 3C shows the percentage change induced by colchicine (300 μ M) of 5-HT (0.5–10 μ M)-mediated currents. Colchicine enhanced 0.5 μ M 5-HT-

Fig. 3. Interaction of colchicine with the human 5-HT_{3A}R. **A**, colchicine enhances currents evoked by 0.5 μM 5-HT in oocytes expressing the human 5-HT_{3A}R. Serotonin (0.5 μM) was applied for 30 s to obtain a baseline current. Colchicine (300 μM) was coapplied with 5-HT (0.5 μM) for 30 s. A potentiation of ~75% of the baseline current was observed in this receptor. Washout of colchicine-induced potentiation was observed with the next application of 5-HT 5 min later. **B**, concentration-response curve for colchicine (10–1000 μM) was generated in oocytes expressing human 5-HT_{3A}Rs ($n = 4-5$). An EC_{50} for colchicine of 226.6 ± 25.1 μM and a Hill coefficient of 2.38 ± 0.64 were determined. **C**, potentiating actions of colchicine at the human 5-HT_{3A}R is 5-HT concentration dependent and microtubule independent. Colchicine (300 μM) was coapplied with 5-HT (0.5, 1, 3, or 10 μM) for 30 s ($n = 3-4$). The effect of colchicine was expressed as a percentage of the baseline current produced by the respective concentration of 5-HT alone.

mediated currents but had no effect on 1 μM 5-HT (~ EC_{20})-evoked currents. At 3 and 10 μM 5-HT, colchicine had inhibitory actions. As a putative positive allosteric modulator, colchicine's enhancement of receptor function would be predicted to decrease as 5-HT concentrations increased. Because probability of opening is increased with higher 5-HT gating concentrations, there is less ability of a positive allosteric drug to increase it further. The inhibition of human receptor function at high concentrations of 5-HT is probably due to an additional inhibitory site on the receptor. We predict that this site has a lower affinity for colchicine than the positive allosteric site. At high concentrations of 5-HT, the positive allosteric modulation of colchicine (300 μM) would be masked, leaving the inhibitory effects to predominate.

To further examine the putative stimulatory and inhibitory colchicine sites on the human 5-HT_{3A} receptor, we performed binding assays. In Fig. 4A, saturation binding experiments were done with the 5-HT₃R competitive antagonist, [³H]BRL-43694; a K_d of 2.58 ± 0.6 nM was obtained. In Fig. 4B, colchicine (25 nM to 1 mM) did not displace [³H]BRL-43694 (2.6 nM). These results also suggest that colchicine does not act at the 5-HT recognition site of the human receptor. We next examined the ability of colchicine to alter the displacement of [³H]BRL-43694 by 5-HT (Table 3). In the absence of colchicine, the 5-HT K_i was 1530 ± 230 nM. Colchicine (300 μM) significantly decreased the K_i value of 5-HT to 738 ± 0.64 nM. In contrast, colchicine (1000 μM)

increased the K_i value of 5-HT to 2940 ± 240 nM. These results suggest that colchicine acts at two possible allosteric sites on the human 5-HT_{3A} receptor.

To test whether colchicine potentiates the function of the human receptor through a microtubule-dependent mechanism, oocytes were incubated in ice-cold MBS for at least 2 h before recording (Table 1). Colchicine (300 μM) enhanced 0.5 μM 5-HT-mediated currents by $90.3 \pm 4.3\%$, which is not significantly different from that ($79.3 \pm 8.7\%$) recorded in oocytes at room temperature. Likewise, colchicine (300 μM)-induced inhibition of 10 mM 5-HT-mediated responses was similar between oocytes incubated for 2 h on ice ($39 \pm 7.9\%$) and oocytes maintained at room temperature ($34 \pm 5.1\%$).

The Species-Selective Colchicine Actions on Mouse and Human 5-HT_{3A}Rs Are Conferred by Their N-Terminal Extracellular Domains. We have determined that colchicine is both a positive allosteric modulator and inhibitor of the human receptor, whereas it is an antagonist of the mouse receptor. Studies using nACh/5-HT₃ receptor chimeras have shown that the ligand binding domains of the receptors are in the N terminus (Eiselé et al., 1993; Craig et al., 2004). To correlate species-differential colchicine actions to defined structural domains of 5-HT_{3A}Rs, we constructed four chimeric receptor cDNAs, as previously published (Zhang et al., 2006). The first chimera was designated H239M and contains the entire N-terminal domain of the human receptor cDNA and the balance of the mouse 5-HT_{3A}R cDNA. The

Fig. 4. Colchicine action at the human 5-HT_{3A}Rs. **A**, [³H]BRL-43694 binding to HWT 5-HT_{3A} receptor-rich membranes. Membranes were incubated for 2 h with increasing concentrations of [³H]BRL-43694 (0.1–23 nM). Total (▼), specific (■) and nonspecific (▲) binding. Nonspecific binding was measured with MDL-7222, and specific binding was calculated by subtracting nonspecific from total binding. Scatchard plot of the binding data is shown. The K_d (2.58 ± 0.5 nM) was calculated as the negative reciprocal of the slope of three to five separate experiments. **B**, colchicine displacement of [³H]BRL-43694 binding to HWT 5-HT_{3A} receptor-rich membranes. Membrane was incubated (2 h) with increasing concentrations of colchicine (25 nM–1 mM) and [³H]BRL-43694 at the $K_d = 2.58$ nM. Total (▼), specific (■), and nonspecific (▲) binding. Nonspecific binding was measured with MDL-7222, and specific binding was calculated by subtracting nonspecific from total binding. No displacement of the radioligand was observed.

second chimera, M244H, is a mirror image of the first and consists of the entire N-terminal domain of mouse receptor cDNA and the balance the human receptor cDNA. The third chimera, M181H239M, is the mouse receptor cDNA in which the distal one third of the N terminus is replaced by human receptor cDNA, and the fourth corresponding human receptor chimera is H176M244H in which the distal one third of the human receptor cDNA is replaced by mouse receptor cDNA.

These chimeric receptors, along with their parental receptors, MWT and HWT, were expressed in *X. laevis* oocytes, and the responses to 5-HT (0.5 μ M) or 5-HT (0.5 μ M) plus colchicine (300 μ M) were measured. Serotonin concentration-

response curves were performed, and EC_{50} values and Hill coefficients for each receptor construct are listed in Table 2. Percentage change induced by colchicine is represented in Fig. 5. In receptors containing the human receptor N terminus, colchicine enhanced 5-HT evoked currents. Potentiation of 64.3 ± 3.5 and $52.8 \pm 5\%$ was observed in HWT and H239M receptors, respectively. Potentiation of $35.3 \pm 3.3\%$ was measured in M181H239M receptors, which suggests that the distal one third of the N terminus is sufficient to confer positive allosteric modulation by colchicine. However, the enhancement produced by colchicine was significantly less in the M181H239M than the HWT receptor, suggesting that residues in the other two thirds of the human receptor N terminus may play a role in determining colchicine's efficacy. Inhibition by colchicine (300 μ M) was produced in receptors containing the mouse receptor N terminus. Inhibition of 91.7 ± 1.8 , 83.4 ± 2.6 , and $91.7 \pm 1.1\%$ was determined in MWT, M244H, and H176M244H receptors, respectively. Thus, replacement of the distal one third of the human receptor with that of the mouse receptor was sufficient to confer colchicine-induced inhibition.

The Distal One Third of the N Termini of 5-HT_{3A}Rs Confer Positive Allosteric Modulation in the Human Receptor and Competitive Antagonism in the Mouse Receptor. To characterize further the role of the distal one third of the N terminus in the species-selective colchicine action, we performed colchicine concentration-response curves in MWT, HWT, H176M244H, and M181H239M receptors. In Fig. 6A, colchicine concentration-response curves were conducted in the presence of 0.5 μ M 5-HT, an EC_{10} for both MWT and H176M244H receptors. Colchicine inhibited 0.5 μ M 5-HT-mediated currents with the same potency in both receptor constructs, suggesting that replacement of the last one third of the N terminus of the human receptor with that of the mouse receptor is sufficient to generate a receptor that is virtually identical to the MWT receptor in its direction of modulation by and sensitivity to colchicine. In Fig. 6B, a representative tracing of the inhibitory action of colchicine (300 μ M) at the H176M244H receptor is shown.

The actions of colchicine at the MWT receptor were compared with those at the M181H239M receptor (Fig. 7A). A 1 μ M 5-HT concentration, which represents approximately an EC_{50} for both receptor constructs, was used. An EC_{50} concentration of 5-HT was chosen for two reasons. In the MWT receptor, 1 μ M 5-HT-mediated currents were predicted to be inhibited in a concentration-dependent manner. In the human receptor, an EC_{50} concentration of 5-HT would not be predicted to produce currents that could be enhanced by colchicine, given that 1 μ M 5-HT (EC_{20}) in the human receptor was unaffected by colchicine (Fig. 3C). If anything, the actions of colchicine on 1 μ M 5-HT-mediated currents in M181H239M might be predicted to be slightly inhibitory, given the inhibition of 45 and 27% of 3 (EC_{85}) and 10 (EC_{100}) μ M 5-HT in HWT receptor (Fig. 3C). In Fig. 7A, the MWT receptor is inhibited by colchicine, with an IC_{50} of 96.5 ± 6 μ M, whereas the M181H239M receptor was unaffected by colchicine (10–500 μ M). These results demonstrate that replacement of the distal one third of the mouse receptor with that of the human receptor is sufficient to remove the inhibitory actions of colchicine in the mouse receptor.

A full concentration-response curve was generated in the

TABLE 3

Serotonin concentration-response curve values for human and mouse 5-HT_{3A}Rs

5-HT _{3A} -R	MWT	M244H	M181H239M	H176M244H	H239M	HWT
5-HT EC ₅₀ (μM)	0.92 ± 0.06	1.22 ± 0.05	1.21 ± 0.03	1.65 ± 0.34	3.9 ± 0.2	1.39 ± 0.06
Hill number	2.8	2.1	3.2	1.8	2.6	2.7

Fig. 5. Amino acid domains in the N termini are involved in species-selective colchicine actions on the mouse and human 5-HT_{3A}Rs. Responses of the wild-type or chimeric receptors to colchicine (300 μM) in the presence of 0.5 μM 5-HT were measured. The schematic representations of the chimeric receptors tested are shown at the bottom of the diagrams. Oocytes expressing wild-type or chimeric receptors were perfused with 5-HT in the absence or presence of colchicine (300 μM). Bar graphs, percentage change by induced by colchicine (300 μM) ($n = 4-21$). One-way analysis of variance demonstrated a significant effect of mutation [$F_{(5,39)} = 244.94$, $p < 0.0001$]. Student-Newman-Keuls post hoc test revealed that the colchicine-induced modulation in M181H239M, H176M244H, M244H, and MWT receptors was significantly different from that in the HWT receptor ($p < 0.001$).

M181H239M receptor in Fig. 7B. Colchicine (25–1000 μM) enhanced 5-HT-mediated currents in the chimeric receptor, with an EC₅₀ of $296.4 \pm 97.4\%$, with a slightly smaller potency and less efficacy than that seen in the HWT receptor. Thus, in the 5-HT_{3A}R, if the distal one third of the N terminus is human-like, potentiating actions of colchicine will be observed. However, amino acids in the first two thirds of the N terminus may play a role in determining colchicine efficacy. Figure 7C is a typical tracing of the allosteric modulatory actions of colchicine (300 μM) in the M181H239M receptor.

Discussion

A number of studies investigating the relationships between members of the superfamily of LGICs and cytoskeleton intermediary proteins suggest that their associations play an

important role in receptor distribution and function (for review, see Antolik et al., 2007; Chen and Olsen, 2007; Sun et al., 2008). In the present study, we investigated colchicine, a chemical compound more commonly known for its microtubule-depolymerizing actions. Previous studies reported that colchicine acts as a competitive antagonist at the Glyα1 and α2 receptors (Machu, 1998) and at GABA_ARs (Bueno and Leidenheimer, 1998). The antagonism at these receptors was independent of the microtubule-depolymerizing function of colchicine. Results from the present study show that colchicine inhibits 5-HT currents at the mouse 5-HT_{3A}R, whereas at the human 5-HT_{3A}R, both potentiation and inhibition are observed. Furthermore, the potency of colchicine at the mouse 5-HT_{3A}R is similar to that seen at the GABA_AR and slightly higher than that at the GlyRα2.

Concordant with results reported for the GlyRα2 and the GABA_ARs, the actions of colchicine at the 5-HT_{3A}R are independent of microtubule depolymerization. Depolymerization of microtubules with colchicine takes at least 1.5 h to reach equilibrium at 30°C (Owells et al., 1972). Therefore, colchicine would not be expected to produce an immediate effect on the function of 5-HT_{3A}Rs if it were working through a microtubule-dependent mechanism. As predicted, colchicine's inhibition of mouse 5-HT_{3A}R function occurred in the absence of preincubation. Other evidence that colchicine acts directly at the mouse 5-HT_{3A}R include experiments conducted under cold temperatures. Oocyte microtubules are known to be completely depolymerized after 2 h of incubation at 4°C (Gard, 1991). In addition, microtubule depolymerization induced by colchicine does not occur at cold temperatures (Wilson et al., 1974). No significant change in colchicine-induced inhibition was observed in 5-HT-mediated currents in oocytes incubated on ice for 2 h. Furthermore, both potentiating and inhibitory effects of colchicine on the human 5-HT_{3A}R were preserved in oocytes incubated on ice for 2 h. Our results do not exclude the possibility that microtubules play a role in the synaptic localization of LGICs; rather, they demonstrate a direct effect of this microtubule-depolymerizing agent on yet another member of the LGIC family.

Another major finding of the present study is that colchicine acts in a dissimilar manner at mouse and human 5-HT_{3A}Rs, despite the fact that they share 84% amino acid identity. In the human receptor, colchicine enhances 5-HT-mediated currents at low 5-HT concentrations and inhibits function at higher 5-HT concentrations. In contrast, colchicine has inhibitory actions at the mouse receptor. Colchicine did not displace [³H]BRL-43694 binding in mouse or human 5-HT_{3A} receptors and, therefore, does not act at the ligand recognition site. In agreement with the binding experiments, colchicine applied in the absence of 5-HT did not produce any detectable currents in mouse or human receptors. We suggest that colchicine acts at an allosteric site to produce opposing actions in human and mouse receptors, i.e., as an agonist and inverse agonist, respectively. Results of our radioligand binding assays strengthen this hypothesis. When

Fig. 6. The distal one third of the N termini of the mouse 5-HT_{3A}R confers colchicine's inhibitory effects. A, colchicine concentration-response curve in the H176M244H receptor was generated and compared with that obtained for the MWT receptor. Serotonin-mediated currents were measured in the absence and presence of colchicine (1–500 μ M) ($n = 3$ –9). A colchicine IC_{50} of 45.72 ± 2.28 μ M and a Hill coefficient of 1.12 ± 0.07 were obtained for the H176M244H receptor, which is similar to that obtained in the MWT receptor. B, representative tracings of currents produced in a single oocyte expressing H176M244H 5-HT_{3A}Rs. Serotonin (0.5 μ M) was applied for 30 s to obtain a baseline current. Serotonin (0.5 μ M) was applied in the presence of colchicine (300 μ M) for 30 s. An inhibition of $\sim 93\%$ of the 0.5 μ M 5-HT-induced baseline current was observed for the receptor. Washout of colchicine inhibition was observed with the next application of 5-HT 5 min later.

Fig. 7. The distal one third of the N termini of the human 5-HT_{3A}Rs confers colchicine's stimulatory effects. A, actions of colchicine (1–500 μ M) were compared in the MWT and M181H239M receptors. In both constructs, 1 μ M 5-HT was used; this concentration is approximately an EC_{50} for both. Replacement of the distal one third of the N terminus of the mouse receptor with that of the human receptor is sufficient to remove inhibitory effects of colchicine (1–500 μ M). An IC_{50} of 96.5 ± 6 μ M, and a Hill slope of 1.4 ± 0.12 were obtained for the MWT receptor. B, colchicine concentration-response curve in the M181H239M receptor was generated and compared with that obtained for the HWT receptor. Serotonin-mediated currents were measured in the absence and presence of colchicine (1–1000 μ M) ($n = 4$ –21). A colchicine EC_{50} of 296.4 ± 97.4 μ M and a Hill coefficient of 1.09 ± 0.59 were obtained for the M181H239M receptor. C, colchicine enhances currents evoked by 0.5 μ M 5-HT in oocytes expressing the M181H239M 5-HT_{3A}R. Serotonin (0.5 μ M) was applied for 30 s to obtain a baseline current. Colchicine (300 μ M) was coapplied with 5-HT (0.5 μ M) for 30 s, and potentiation of 5-HT-mediated currents was observed. Washout of colchicine-induced potentiation was observed with the next application of 5-HT 5 min later.

colchicine (300 and 1000 μ M) was coapplied with 5-HT, a significant decrease in the potency of 5-HT at the MWT-A receptor was observed. However, in the HWT-A receptor, 300 μ M coapplication of colchicine resulted in a significant in-

crease in 5-HT potency, whereas 1000 μ M coapplication of colchicine led to a significant decreased in 5-HT potency (Table 2). These findings parallel the bidirectional modulation of GABA_AR function by ligands binding to the BZD

recognition site (for review, see Sigel and Buhr, 1997). BZD analogs are agonists, antagonists, or inverse agonists, but we are unaware of any single compound subserving a function as agonist in one GABA_AR subunit combination and inverse agonist in another.

Further examination of the potentiating actions of colchicine at the human 5-HT_{3A}R revealed that potentiation decreased as the concentration of coapplied 5-HT increased, which is similar to results obtained with BZD agonists at the GABA_AR. However, unlike BZD's actions at the GABA_AR, when coapplied with 5-HT concentrations >1 μ M, colchicine acted as an inhibitor at the human receptor, thus suggesting that a second inhibitory allosteric site may exist in the human 5-HT_{3A}R. If a second site exists in the mouse receptor, it would have a lower affinity than the human receptor for colchicine, based on the lack of inhibitory action of colchicine (10–500 μ M) at mouse chimeric receptor M181H239M. Such a putative site could exist in any part of the receptor, but a channel binding domain is doubtful, given that the transmembrane (TM) two domains that contain the pore-lining residues are identical between the two species.

A major goal of the present study was to investigate the amino acid domains responsible for the switch between positive allosteric modulation in the human 5-HT_{3A}R and inverse agonism in the mouse receptor by colchicine. The agonist binding domain of Cys-loop receptors is located in the N terminus at the interface of two subunits. Loops A to C are in the principal component of the LBD, and loops D to F are on the complementary face of the binding domain (Brejc et al., 2001). In addition to agonist ligands, allosteric modulators are also known to bind to the N-terminal domain of Cys-loop receptors, with the best known example being BZDs, which bind to the interface of α/γ subunits of GABA_ARs (Sigel, 2002). Loops C and F have been reported to play a critical role in differential pharmacological actions of drugs on both 5-HT_{3A} and GABA_ARs (Zhang et al., 2006, 2007; Padgett and Lummis, 2008). There are 16 differences between mouse and human receptors in the distal one third of the N terminus, seven in loop C, and nine in or within proximity of loop F. To explore the N terminus as the potential location for the colchicine binding site, we created mouse-human and human-mouse chimeric receptors. The first set of chimeras consisted of whole N-terminal substitutions. The function of the mouse-human chimera, in which the N terminus was mouse-like, was inhibited by colchicine. In contrast, the function of the mirror image human-mouse chimera receptor was enhanced by colchicine. The distal one third of the N terminus was substituted in the second set of chimeras. Substitution of the distal one third of the human receptor with that of the mouse yielded a chimera, H176M244H, whose function was inhibited by colchicine in the presence of 0.5 μ M 5-HT; the IC₅₀s were similar for H176M244H and MWT. The converse effects were observed in the mirror image chimera, M181H239M, in which the distal one third of the N terminus of the mouse receptor was replaced with that of the human receptor. These results suggest that the site responsible for colchicine's allosteric modulation of function at low gating concentrations of 5-HT is located in the distal one third of the N termini of mouse and human 5-HT_{3A} receptors.

The underlying mechanism through which loops C and/or F determine the direction of allosteric modulation of colchicine in mouse and human 5-HT_{3A}Rs remains to be deter-

mined. Our binding and electrophysiological studies demonstrated that colchicine does not compete with [³H]BRL-43694 and 5-HT, respectively, at the 5-HT recognition site. Because every subunit interface is identical in the 5-HT_{3A}R, colchicine and BRL-43694 would be expected to compete, should they both bind at the junction of two subunit interfaces. Thus, these results strongly suggest that amino acid differences in the N terminus of mouse and human 5-HT_{3A}Rs exert their differential actions by affecting gating, not binding. These loops also have been shown to play a significant role in modulation of GABA-induced currents by BZDs and other drugs binding at the BZD binding site on the GABA_AR (Sancar et al., 2007; Padgett and Lummis, 2008). The role of loops C and F in the GABA-BZD modulatory mechanism differs from the proposed role of these loops in colchicine modulation in that loops C and F probably induce conformational changes that affect gating of the 5-HT_{3A}R. In the GABA_ARs, these loops are thought to not only affect gating but also to play a direct role in stabilization of the BZD binding site. The binding site(s) for colchicine in the mouse and human 5-HT_{3A} receptors could be in regions of the N terminus that do not form the ligand binding domains or in the transmembrane domains. Residues in TM1 and TM2 domains of the $\alpha 7$ nAChR have been identified recently as strong candidates for binding of positive allosteric modulators (Young et al., 2008).

A number of clinical compounds targeting the 5-HT_{3A}R are currently in use (for review, see Thompson and Lummis, 2007). A better understanding of the mechanisms underlying binding, gating, and allosteric modulation of this receptor can potentially advance drug development. The data from this study further support existing evidence of a critical role for loops C and F in function of the 5-HT_{3A}R and introduce colchicine as a powerful tool in dissecting the molecular interactions in the 5-HT_{3A}R that are responsible for receptor modulation.

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