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The 5-HT_{3B} Subunit Confers Spontaneous Channel Opening and Altered Ligand Properties of the 5-HT_3 Receptor*[S]

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Current receptor theory suggests that there is an equilibrium between the inactive (R) and active (R*) conformations of ligand-gated ion channels and G protein-coupled receptors. The actions of ligands in both receptor types could be appropriately explained by this two-state model. Ligands such as agonists and antagonists affect receptor function by stabilizing one or both conformations. The 5-HT_3 receptor is a member of the Cys-loop ligand-gated ion channel superfamily participating in synaptic transmission. Here we show that co-expression of the 5-HT_{3A} and 5-HT_{3B} receptor subunits in the human embryonic kidney (HEK) 293 cells results in a receptor that displays a low level of constitutive (or agonist-independent) activity. Furthermore, we also demonstrate that the properties of ligands can be modified by receptor composition. Whereas the 5-hydroxytryptamine (5-HT) analog 5-methoxyindole is a partial agonist at the 5-HT_{3A} receptor, it becomes a "protean agonist" (functioning as an agonist and an inverse agonist at the same receptor) at the 5-HT_{3AB} receptor (after the Greek god Proteus, who was able to change his shape and appearance at will). In addition, the 5-HT analog 5-hydroxyindole is a positive allosteric modulator for the liganded active (AR*) conformation of the 5-HT_{3A} and 5-HT_{3AB} receptors and a negative allosteric modulator for the spontaneously active (R*) conformation of the 5-HT_{3AB} receptor, suggesting that the spontaneously active (R*) and liganded active (AR*) conformations are differentially modulated by 5-hydroxyindole. Thus, the incorporation of the 5-HT_{3B} subunit leads to spontaneous channel opening and altered ligand properties.

LGICs[2] are transmembrane proteins that mediate synaptic transmission upon binding of neurotransmitters at the chemical synapse. All of the LGICs are formed by the assembly of homologous subunits. The 5-hydroxytryptamine type 3 (5-HT_3) receptor, a member of the Cys-loop LGIC superfamily, which includes nicotinic acetylcholine, γ-aminobutyric acid, type A ($GABA_A$), and glycine receptors (1), can exist in either homomeric (5-HT_{3A}) or heteromeric (5-HT_{3AB}) forms. Although the 5-HT_{3B} receptor subunit itself cannot form functional channels, when co-expressed with the 5-HT_{3A} receptor subunit it alters biophysical properties (2–4) and reduces allosteric modulation by alcohol and anesthetics (5–7). In the absence of agonist, channel opening of the 5-HT_{3A} receptor is theoretically possible but has not been observed experimentally. However, certain mutations in the 5-HT_{3A} receptor, such as 5-HT_{3A} V13′S (V291S), have produced spontaneously opening channels (8, 9). The constitutive activity of the 5-HT_{3A} V13′S mutant can be enhanced by incorporation of the 5-HT_{3B} subunit (9), which may indicate that the heteromeric 5-HT_{3AB} receptor is able to open spontaneously in the absence of agonist.

LGICs exist in two general classes of states: inactive (closed) and active (open), and each of these classes may contain multiple states that differ in average lifetime, agonist site occupancy, etc. The function of LGICs is determined by the transitions among these states. However, the mechanism for receptor activation is not completely understood. Based on observations on ligand-gated ion channels, a two-state model of receptor activation was proposed by del Castillo and Katz in 1957 (10) (Fig. 1). The del Castillo-Katz mechanism represents a sequential model, which suggests that the receptor is inactive in the absence of agonist and the binding of agonist to the inactive state (R) induces a conformational change of the receptor to an activated state (R*). Monod, Wyman, and Changeux (1965) proposed that the oligomeric proteins can undergo reversible transitions between discrete conformations such as R ↔ R* in the absence of agonist (11). This concept was supported by the finding of constitutive activity in GPCRs first reported by Costa and Herz in 1989 (12). Because the del Castillo-Katz mechanism does not predict spontaneous channel opening in the absence of agonist, a modified two-state model was established (Fig. 1) (13). According to this model, receptors exist in a conformational equilibrium between R and R* of the unliganded receptor; and compounds alter receptor function by causing a re-distribution between the two states. An agonist preferentially binds and stabilizes the R* conformation, and an inverse agonist preferentially binds and stabilizes the R conformation. A neutral antagonist binds equally to both conformations. Although this allosteric two-state model could adequately explain most receptor behavior, it remains unknown whether the spontaneously active (R*) and liganded active (AR*) conformations are the same and whether AR* conformations are the same for different agonists (14).

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4.

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[2] The abbreviations used are: LGIC, ligand-gated ion channel; 5-HT, 5-hydroxytryptamine; 5-MI, 5-methoxyindole; 5-HI, 5-hydroxyindole; $GABA_A$, γ-aminobutyric acid, type A; GPCR, G protein-coupled receptor.

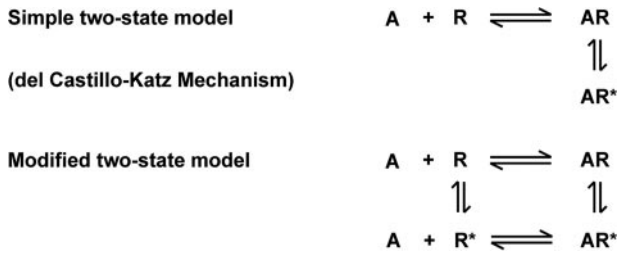

FIGURE 1. **Two-state models of receptor activation by agonists.**

EXPERIMENTAL PROCEDURES

Complementary DNA Constructs and Transfection—5-HT$_{3A}$ (gift from Dr. D. Julius) and 5-HT$_{3B}$ (gift from Dr. E. F. Kirkness) receptor subunits were subcloned into the vector pcDNA3.1 (Invitrogen). HEK 293 cells were transiently transfected with the 5-HT$_{3A}$ or 5-HT$_{3A}$ and 5-HT$_{3B}$ receptor cDNA using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Green fluorescent protein (pGreen Lantern, Invitrogen) was co-expressed with the 5-HT$_3$ receptor subunits to permit selection of transfected cells under fluorescence optics.

Patch Clamp Electrophysiology—Whole-cell patch clamp recordings were performed as described previously (15), in HEK cells transfected with 5-HT$_3$ receptors and green fluorescent protein. The external solution contained (in mM): 140 NaCl, 5 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4 with NaOH and ~340 mosmol liter^{-1} with sucrose). Patch pipettes contained (in mM) 140 CsCl, 2 $MgCl_2$, 10 EGTA, and 10 HEPES (pH 7.2 with CsOH and ~315 mosmol liter^{-1} with sucrose).

Data Analysis—Data were acquired using pCLAMP 9.0 software (Axon). Currents were filtered at 2 kHz and digitized at 5–10 kHz. Data analysis and curve-fitting were performed with Origin 7.0 (Microcal), pCLAMP 9.0 (Axon), or GraphPad InStat 3.0 (GraphPad) software. Data are presented as mean ± S.E.

RESULTS

Incorporation of the 5-HT$_{3B}$ Subunit Alters Desensitization Kinetics of the 5-HT$_3$ Receptor—The heteromeric 5-HT$_{3AB}$ receptor was transiently expressed in the HEK 293 cells, and the successful incorporation of the 5-HT$_{3B}$ receptor subunit was confirmed by examining the desensitization kinetics. The transfected cells were exposed to a maximally efficacious concentration of 5-HT (30 μM) for 10 s. In the continued presence of the agonist the 5-HT current decayed after reaching a peak in the 5-HT$_{3A}$ and 5-HT$_{3AB}$ receptors, a process representing receptor desensitization (Fig. 2*A*). The desensitization was gradual and monoexponential in the 5-HT$_{3A}$ receptor. However, the current decay was much faster and biexponential in the 5-HT$_{3AB}$ receptor. The overall desensitization measured as weighted desensitization was ~6-fold faster in the 5-HT$_{3AB}$ receptor than in the 5-HT$_{3A}$ receptor (Fig. 2*B*).

Spontaneous Channel Opening of the 5-HT$_{3AB}$ Receptor Is Conferred by the 5-HT$_{3B}$ Subunit—The possibility of spontaneous opening of the 5-HT$_{3AB}$ receptor was examined by exposing the transfected cells to the 5-HT$_3$ receptor antagonist MDL 72222 and 5-HT analogs 5-methoxyindole (5-MI) and 5-hydroxyindole (5-HI) (see supplemental Fig. 1 for chemical structures). We first tested the actions of the 5-HT$_3$ receptor antagonist MDL 72222 (Fig. 3*A*). MDL 72222 was previously used to detect spontaneous channel openings of Arg-246 mutant 5-HT$_{3A}$ receptors (8). Consistent with previous studies (15, 16), at a holding potential of −60 mV, MDL 72222 (1 μM) did not alter the base-line current at the 5-HT$_{3A}$ receptor. However, an application of MDL 72222 produced a decrease in the base-line current (manifesting as an apparent outward current) at the 5-HT$_{3AB}$ receptor that was 1.2 ± 0.4% of the amplitude of the 30 μM 5-HT-activated response. This is consistent with a low level of constitutive activity of the 5-HT$_{3AB}$ receptor due to spontaneous opening of the channels. The apparent outward current persisted for more than 20 s after removal of MDL 72222, which is most probably the result of the high affinity of MDL 72222 for the receptor. We also used Zn^{2+} (1 mM), an open channel blocker at the 5-HT$_3$ receptor,[3] to examine constitutive activity of the 5-HT$_3$ receptors (Fig. 3*B*). Co-application of Zn^{2+} with 5-HT rapidly brought 5-HT-activated current to base-line levels in the 5-HT$_{3A}$ receptor due to blocking of the open channels. Interestingly, Zn^{2+} produced an overshoot above the base line during application of 5-HT in the 5-HT$_{3AB}$ receptor, suggesting that the activity of the 5-HT$_{3AB}$ receptor is suppressed below its spontaneous opening state. This overshoot current provides further evidence for the existence of constitutive activity of the 5-HT$_{3AB}$ receptor.

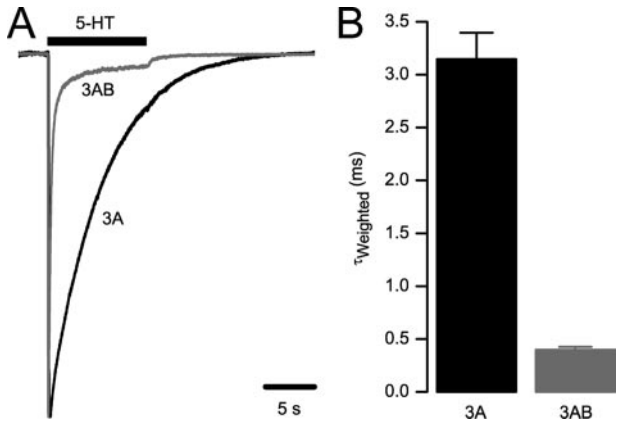

FIGURE 2. **Altered desensitization kinetics with the incorporation of the 5-HT$_{3B}$ receptor subunit.** *A*, whole-cell currents evoked by 30 μM 5-HT for 10 s in HEK 293 cells expressing the 5-HT$_3$ receptors show the desensitization of the 5-HT$_{3A}$ and 5-HT$_{3AB}$ receptors at a holding potential of −60 mV. Currents were normalized to peak current and superimposed to compare the desensitization kinetics between the 5-HT$_{3A}$ (*black*) and 5-HT$_{3AB}$ (*gray*) receptors. *B*, *bar graph* summarizes the overall desensitization kinetics.

5-HT Analogs Reveal Distinct Active Conformations of the 5-HT$_{3AB}$ Receptor—The use of 5-HT analogs (supplemental Fig. 1) also revealed the constitutive activity of the 5-HT$_{3AB}$ receptor. The 5-HT analog 5-MI functioned as a partial agonist at the 5-HT$_{3A}$ receptor (Fig. 4*A*). A rebound current appeared upon termination of 5-MI application. The maximal response activated by 5 mM 5-MI was ~34.4 ± 3.3% of the response activated by 30 μM 5-HT at −60 mV. At the 5-HT$_{3AB}$ receptor 5-MI displayed unusual behavior. 5-MI (5 mM) evoked a transient inward current followed by an apparent outward current (Fig. 4*A*). The amplitude of the inward current was 3.5 ± 0.7% of that activated by 30 μM 5-HT at −60 mV, which is signifi-

[3] X.-Q. Hu, unpublished data.

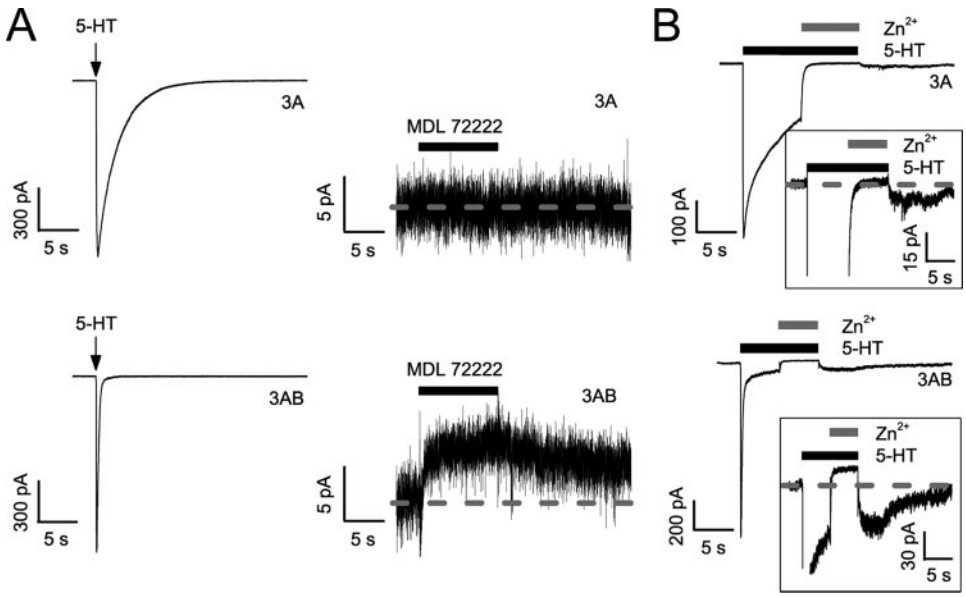

FIGURE 3. **Constitutive activity in the 5-HT$_{3AB}$ receptor is determined by the 5-HT$_{3B}$ receptor subunit.** *A*, current traces show the responses elicited by 30 μM 5-HT (*left*) and 1 μM MDL 72222 (*right*) alone in HEK 293 cells expressing the 5-HT$_{3A}$ and 5-HT$_{3AB}$ receptors. Drug applications were separated by 2-min intervals to ensure complete drug washout and the return of the current to base-line. The *dashed line* indicates the base-line current level. *B*, current traces show the actions of Zn^{2+} (1 mM) on 30 μM 5-HT-activated responses in the 5-HT$_{3A}$ and 5-HT$_{3AB}$ receptors. *Insets*, actions of Zn^{2+} with expanded scales. The *dashed line* indicates the base-line current level.

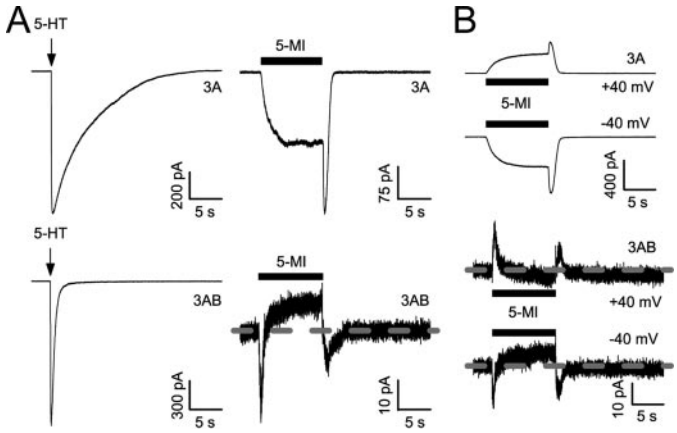

FIGURE 4. **Incorporation of the 5-HT$_{3B}$ receptor subunit alters the properties of the 5-HT analog 5-MI.** *A*, current traces show the responses elicited by 30 μM 5-HT (*left*) and 5 mM 5-MI alone (*right*) in HEK 293 cells expressing the 5-HT$_{3A}$ and 5-HT$_{3AB}$ receptors at a holding potential of −60 mV. The drug application protocol was similar to that described in Fig. 3*A*. *B*, current traces show the effect of holding potentials on 5-MI-evoked currents in the 5-HT$_{3A}$ and 5-HT$_{3AB}$ receptors. The *dashed line* indicates the base-line level.

cantly reduced when compared with the 5-HT$_{3A}$ receptor ($p <$ 0.01, Student's t test). This finding suggests that the efficacy of 5-MI is reduced in the 5-HT$_{3AB}$ receptor. The amplitude of the apparent outward current was 0.9 ± 0.1% of 30 μM 5-HT-activated response. This action of 5-MI was readily reversible, and there was also a rebound current upon removal of 5-MI. The characteristics of 5-MI activation at negative and positive holding potentials were similar in the 5-HT$_{3A}$ receptor and in the 5-HT$_{3AB}$ receptor (Fig. 4*B*), with the exception that the direction of the currents was reversed. The observation that 5-MI produced both a transient inward current and a persistent apparent outward current at negative holding potentials is consistent with both positive and negative (inverse) agonism at the 5-HT$_{3AB}$ receptor.

We also explored the actions of 5-HI, an analog of 5-HT that is a positive allosteric modulator of the 5-HT$_3$ receptor (17, 18). 5-HI (5 mM), when co-applied with 5-HT (30 μM) following a delay of 250 ms or 10 s, enhanced the current amplitude by 17.1 ± 1.3% and 14.5 ± 1.0%, respectively, in the 5-HT$_{3A}$ receptor (Fig. 5*A*). Similar applications of 5-HI during the course of agonist application enhanced agonist-activated steady-state current by 66.7 ± 7.8% for 5-HT and 53.7 ± 1.5% for dopamine (a partial agonist) in the 5-HT$_{3AB}$ receptor (Fig. 5*B*). A pure positive allosteric modulator facilitates agonist-mediated receptor activity without intrinsic agonism. As expected, 5-HI alone did not change the base-line current in the 5-HT$_{3A}$ receptor in the absence of agonist. Unlike 5-MI, 5-HI did not activate a transient inward current but only induced an apparent outward current in the 5-HT$_{3AB}$ receptor. This apparent outward current was rapidly reversible upon removal of 5-HI (Fig. 5*C*) and had an amplitude that was 1.1 ± 0.3% of that activated by 30 μM 5-HT. Therefore, 5-HI selectively produces negative (inverse) agonism but not the positive agonism seen with 5-MI in the 5-HT$_{3AB}$ receptor. As was the case for 5-MI, there was a rebound current upon the removal of 5-HI. The occurrence of the rebound current has also been observed for other negative allosteric modulators or inverse agonists such as bicuculline at the $\alpha1\beta3\gamma2$L GABA$_A$ receptor (19), 17α-methyltestosterone at the $\alpha2\beta3\epsilon$ GABA$_A$ receptor (20), and strychnine at the D97R glycine receptor (21). The nature of the rebound current is not clear at present; it likely represents a rapid unbinding of 5-MI or 5-HI from a low affinity site.

The other 5-HT analog 3-(2-hydroxyethyl)indole acted as an agonist in both the 5-HT$_{3A}$ and 5-HT$_{3AB}$ receptor, although the relative efficacy of 3-(2-hydroxyethyl)indole was dramatically reduced by the incorporation of the 5-HT$_{3B}$ subunit (17.0 ± 1.3% → 2.0 ± 0.4%; supplemental Fig. 2). This observation implies that the hydroxyl (for 5-HI) or methoxyl (for 5-MI) group at position 5 of the indole molecule is required for detecting spontaneous activity of the 5-HT$_{3AB}$ receptor.

Properties of the Constitutive Activity of the 5-HT$_{3AB}$ Receptor—The nature of the 5-HI current was further examined by studying the current-voltage (I-V) relationship and reversal potential. The I-V relationship of the 5-HI current is symmetrical to that of 5-HT current in the 5-HT$_{3AB}$ receptor. Whereas the 5-HT current showed inward rectification, the 5-HI current displayed apparently outward rectification

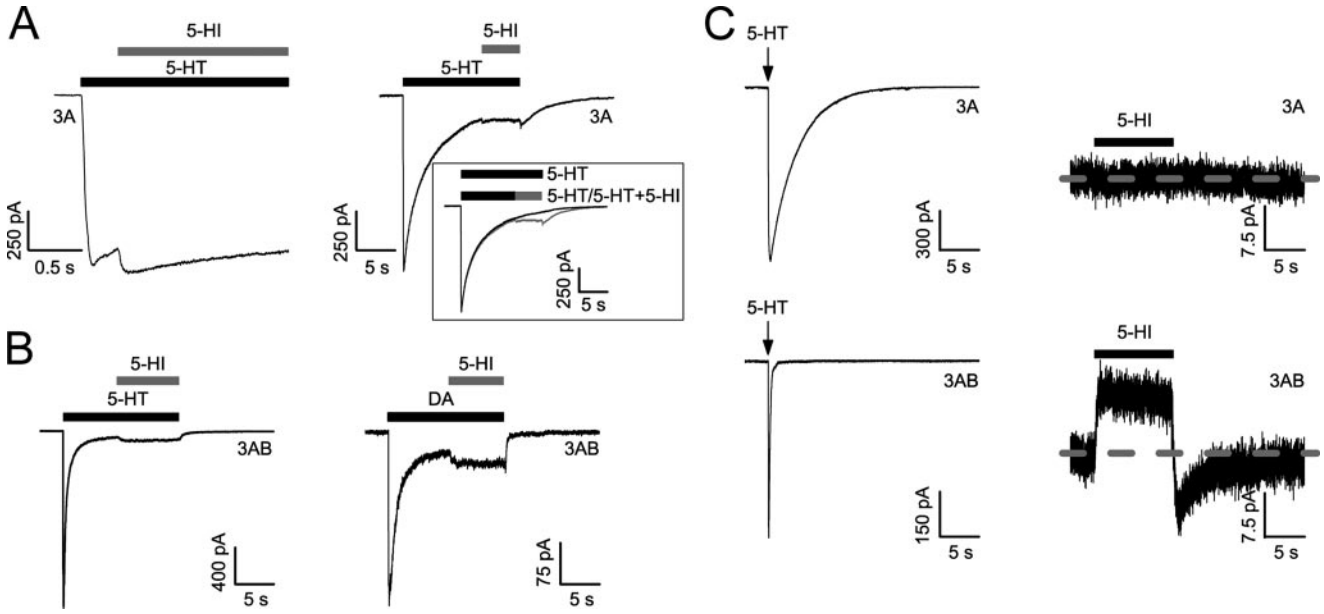

FIGURE 5. **Effects of the 5-HT analog 5-HI on liganded and unliganded active states.** *A*, current traces show 5-HI enhancement of 5-HT-evoked currents in HEK 293 cells expressing the 5-HT$_{3A}$ receptor. *Insets*, superimposed traces showing the potentiation of 5-HT response by 5-HI. *B*, current traces show 5-HI enhancement of 5-HT- and dopamine (*DA*)-evoked currents in the HEK 293 cells expressing 5-HT$_{3AB}$ receptor. *C*, current traces show the responses elicited by 30 μM 5-HT (*left*) and 5 mM 5-HI alone (*right*) in the 5-HT$_{3A}$ and 5-HT$_{3AB}$ receptors. The drug application protocol was similar to that described in Fig. 3*A*. The *dashed line* indicates the base-line current level.

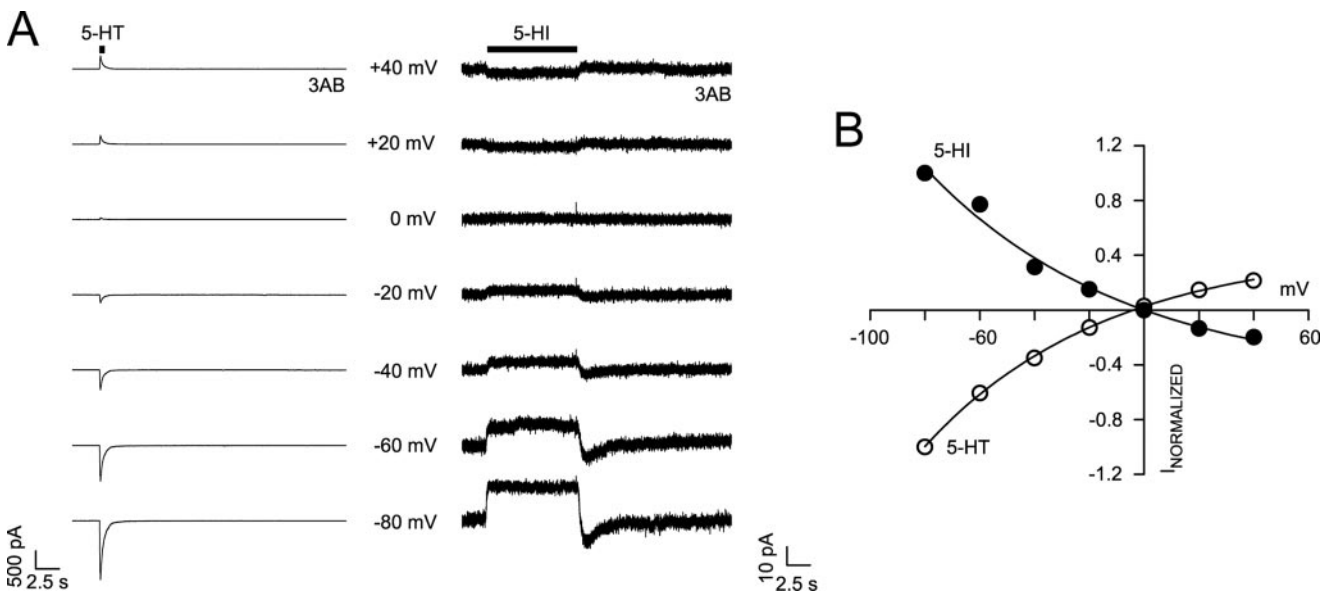

FIGURE 6. **5-HI current is mediated by the 5-HT$_{3AB}$ receptor.** *A*, current traces evoked by 3 μM 5-HT and 5 mM 5-HI at different holding potentials in HEK 293 cells transfected with the 5-HT$_{3AB}$ receptor. *B*, current-voltage (I-V) relationship for 5-HT- and 5-HI-evoked currents. Data are normalized to the current amplitude obtained at −80 mV. Similar results were obtained from 4 to 5 cells.

(Fig. 6). The 5-HI current reversed at 3.3 ± 0.4 mV, which is comparable to the reversal potential for 5-HT at the 5-HT$_{3AB}$ receptor (2.9 ± 0.3 mV). Those observations suggest that the constitutive activity is mediated by the 5-HT$_{3AB}$ receptor and that both the spontaneously active and ligand-activated receptors have the same ionic permeability. The onset of blocking the spontaneously active channel displayed 5-HI concentration dependence, whereas the offset of blocking was largely independent of 5-HI concentrations (supplemental Fig. 3, *A* and *B*). The rate of blocking ($1/\tau_{Block}$) is linear with 5-HI concentration with a slope of 5.9×10^4 $M^{-1}s^{-1}$ (k_{on}, 5-HI binding rate, supplemental Fig. 3*C*). The *y*-axis intercepts were similar for linear regression fitting of $1/\tau_{Block}$ and $1/\tau_{Unblock}$, being 4.5 s^{-1} and 4.8 s^{-1}, respectively (k_{off}, 5-HI unbinding rate). Therefore, the equilibrium dissociation constant (K_i) for 5-HI blocking the spontaneous channel opening is ~7.5 mM.

DISCUSSION

Most GPCRs and LGICs exhibit a low to negligible level of constitutive activity. However, the level of constitutive activity can be enhanced by mutations and overexpression (14, 22, 23).

In addition, certain receptor subunit combinations in LGICs exhibit constitutive activity (24). In the 5-HT$_{3A}$ receptor the energy barrier for channel opening is sufficiently high to prevent spontaneous openings in the absence of agonist. The shifts of base-line current by MDL 72222 in the absence of agonist and Zn^{2+} in the presence of agonist support the existence of constitutive activity in the 5-HT$_{3AB}$ receptor. The 5-HT$_{3B}$ subunit does not form functional homomeric receptors, but the observations in this study indicate that it has a lower energy barrier to channel opening relative to the 5-HT$_{3A}$ subunit. It is not currently clear how structural constraints keep the unliganded 5-HT$_{3A}$ receptor in the inactive conformation and how the incorporation of the 5-HT$_{3B}$ receptor subunit disrupts these structural constraints. The amino acid sequence of the 5-HT$_{3B}$ receptor subunit has only ~45% homology with the 5-HT$_{3A}$ receptor subunit (2, 3). A recent study using atomic force microscopy has revealed that the 5-HT$_{3AB}$ receptor is formed by two 5-HT$_{3A}$ subunits and three 5-HT$_{3B}$ receptor subunits (25). The conformational change associated with ion channel opening in 5-HT$_3$ receptors is proposed to involve a rotation of the M2 helices (26). Although the precise number of subunits that must undergo the conformational change to open the channel is unclear at present, results from nicotinic acetylcholine receptors would suggest that two activated subunits may be sufficient to open the channel (27). Constitutive activity is determined by the equilibrium between the two interconvertible conformations R and R*. If the 5-HT$_{3B}$ subunit is indeed able to spontaneously undergo this conformational change, the stoichiometry of the 5-HT$_{3AB}$ receptor (B-B-A-B-A) would thus make it likely that some channels could spontaneously open in the absence of agonist due to altering the equilibrium constant between R and R*.

Since the description of the constitutive activity in a GPCR by Costa and Herz in 1989 (12), a variety of compounds originally described as antagonists have been shown to exhibit effects opposite to those of agonists (*i.e.* suppressing constitutive activity) and have thus been reclassified as inverse agonists. The observation that MDL 72222, a traditional 5-HT$_3$ receptor antagonist, inhibits constitutive activity suggests that this compound could function as an inverse agonist at the 5-HT$_{3AB}$ receptor. In addition, based on theoretical grounds, Kenakin (23, 28) proposed that positive agonism could revert to negative (inverse) agonism and referred to this as "protean agonism" after the Greek god Proteus who could change his shape and appearance at will. This phenomenon has also been termed as functional selectivity, agonist-directed trafficking of receptor stimulus, and biased agonism (29). A protean agonist is a positive agonist in a quiescent system and an inverse agonist in a constitutive system. GPCRs are usually linked to multiple signal transduction pathways, so that a protean agonist could selectively enhance one or more pathways but depress the others. The existence of compounds that exhibit protean agonism was later confirmed in GPCRs (30). In contrast to GPCRs, LGICs are coupled to a single ion-permeation pathway, and protean agonism has not been observed. Our observations in the present study confirm the existence of protean agonism in LGICs.

The 5-HT$_{3A}$ receptor represents a quiescent receptor system because it does not exhibit constitutive activity. Therefore, in the 5-HT$_{3A}$ receptor 5-MI is a classical del Castillo-Katz agonist that enhances the R → AR* transition. However, the R and R* conformations co-exist in the 5-HT$_{3AB}$ receptor. According to the two-state model, 5-MI could bind to both forms and enhance both the R → AR* and R* → AR* transitions leading to agonism. Furthermore, 5-MI, perhaps by an action at an additional site, could also promote the R* → R transition to produce an inverse agonism. Therefore, both agonism and inverse agonism (*i.e.* protean agonism) could be observed for 5-MI at the 5-HT$_{3AB}$ receptor. Mutations in the transmembrane 2 domain (at the 12′ position) of the muscle nicotinic acetylcholine receptor create spontaneously opening channels that are accompanied by a conformational change at the binding sites (31). Such a conformational change might prevent the agonist from binding to the binding pocket and impede the R* → AR* isomerization. The actions of 5-MI in the 5-HT$_{3AB}$ receptor seem to be consistent with this scheme. The major event for receptor activation would then still follow the del Castillo-Katz mechanism even in the constitutively active system (supplemental Fig. 4). Here we present evidence showing that 5-MI operates as an agonist in the 5-HT$_{3A}$ receptor and an agonist and an inverse agonist in the 5-HT$_{3AB}$ receptor. It is unlikely that a ligand binds to the same site to induce different conformational changes. We propose that the agonism and inverse agonism of 5-MI are mediated by binding to two distinct sites.

According to the two-state model, an agonist has high affinity for the R* conformation and shifts the balance to the R* conformation. Hence, the R* conformation is assumed to be the same as the AR* conformation. Here we demonstrate that 5-MI displays both agonism and inverse agonism at the 5-HT$_{3AB}$ receptor. Furthermore, our results reveal that 5-HI is a positive modulator when the receptor is in the AR* conformation and a negative modulator (inverse agonist) when the receptor is in the R* conformation (supplemental Fig. 4). If the R* and AR* share the same conformation we would expect an enhanced base-line current by 5-HI in the absence of agonist in the 5-HT$_{3AB}$ receptor. It is likely that 5-HI may interact with discrete sites to exert different efficacies leading to alterations in the transition equilibrium between states. The other possible explanation to our observations is that R* and AR* may represent two distinct conformations. This notion needs to be explored in future studies.

5-HT itself is an agonist at 5-HT$_3$ receptors. However, our observations that 5-HT analogs such as 5-MI and 5-HI can selectively modulate different active conformations in the 5-HT$_3$ receptor suggest that subtle changes in chemical structure of an agonist can dramatically alter the functional properties. Therefore, modifications of the structure of 5-HT, 5-HI, and 5-MI may generate useful tools to study the 5-HT$_3$ receptor mechanism and ligand-receptor interaction and could lead to the development of therapeutic agents that selectively act on specific kinetic states of the 5-HT$_3$ receptor. In addition to constitutive activity, receptors can also be tonically activated by low concentrations of ligands such as neurotransmitters and hormones *in vivo* (32). Therefore, the positive and negative allosterism of 5-HI can also be a useful tool to distinguish constitutive activity from tonic activity. 5-MI and 5-HI are a partial agonist and a positive allosteric modulator, respectively, in the 5-HT$_{3A}$ receptor. The incorporation of the 5-HT$_{3B}$ receptor

subunit results in the altered properties of these two ligands. Therefore, the subunit composition of the 5-HT$_3$ receptor could regulate its biophysical and pharmacological properties. Taken together, our results suggest that the activity profile of a ligand is determined by the properties of both the ligand and receptor.

In summary, our study demonstrates that the 5-HT$_{3B}$ receptor subunit imparts constitutive receptor activity to the 5-HT$_3$ receptor. The occurrence of the constitutive activity leads to alterations in the pharmacological properties of 5-HT analogs. These findings provide new insights into the receptor activation mechanism and may hold promise for the development of new therapeutic agents.

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