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The L293 residue in transmembrane domain 2 of the 5-HT_{3A} receptor is a molecular determinant of allosteric modulation by 5-hydroxyindole

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ABSTRACT

Allosteric modulation of ligand-gated ion channels can play important roles in shaping synaptic transmission. The function of the 5-hydroxytryptamine (serotonin) type 3 (5-HT_3) receptor, a member of the Cys-loop ligand-gated ion channel superfamily, is modulated by a variety of compounds such as alcohols, anesthetics and 5-hydroxyindole (5-HI). In this study, the molecular determinants of allosteric modulation by 5-HI were explored in N1E-115 neuroblastoma cells expressing the native 5-HT_3 receptor and HEK 293 cells transfected with the recombinant 5-HT_{3A} receptor using molecular biology and whole-cell patch-clamp techniques. 5-HI potentiated 5-HT-activated currents in both N1E-115 cells and HEK 293 cells, and significantly decreased current desensitization and deactivation. Substitution of Leu293 (L293, L15′) in the second transmembrane domain (TM2) with cysteine (L293C) or serine (L293S) abolished 5-HI modulation. Other mutations in the TM2 domain, such as D298A and T284F, failed to alter 5-HI modulation. The L293S mutation enhanced dopamine efficacy and converted 5-HI into a partial agonist at the mutant receptor. These data suggest that 5-HI stabilizes the 5-HT_{3A} receptor in the open state by decreasing both desensitization and 5-HT unbinding/channel closing; and L293 is a common site for both channel gating and allosteric modulation by 5-HI. Our observations also indicate existence of a second 5-HI recognition site on the 5-HT_{3A} receptor, which may overlap with the 5-HT binding site and is not involved in the positive modulation by 5-HI. These findings support the idea that there are two discrete sites for 5-HI allosteric modulation and direct activation in the 5-HT_{3A} receptor.

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1. Introduction

Ligand-gated ion channels constitute a group of membrane-bound proteins that control the flux of ions across the cell membrane. The 5-hydroxytryptamine (serotonin) type 3 (5-HT_3), nicotinic acetylcholine (nACh), γ-aminobutyric acid type A ($GABA_A$), and glycine receptors belong to the Cys-loop ligand-gated ion channel superfamily, which mediate synaptic transmission and modulate neurotransmitter release (Ortells and Lunt, 1995; Reeves and Lummis, 2002). Members of the superfamily are pentamers formed by assembly of one (homomeric) or different (heteromeric) subunits. The subunits that form these receptors share similar topology, with each subunit possessing an extracellular N-terminal domain, four transmembrane domains (TMs) and loops connecting those TMs. The interfaces between subunits formed by the extracellular N-terminal domains comprise the agonist binding sites. The TM2 domains form the pore, which is surrounded by an outer protein shell comprising the other TMs.

The 5-HT_3 receptor participates in a variety physiological functions, such as cognitive processing, sensory transmission, regulation of autonomic function, integration of the vomiting reflex, pain processing and control of anxiety (Barnes and Sharp, 1999). Thus, agents that alter 5-HT_3 receptor function, including allosteric modulators, are of interest because altering receptor function could be a useful therapeutic strategy for diseases associated with the receptor. The 5-HT_3 receptor is not only the target of the natural agonist 5-HT but also a variety of allosteric modulators including the 5-HT analog 5-hydroxyindole (5-HI). Both 5-HT and 5-HI are metabolites of tryptophan (Mannaioni et al., 2003). 5-HI has been shown to increase the release of neurotransmitter in the cerebellum and hippocampus (Zwart et al., 2002; Mannaioni et al., 2003) and triggers convulsions in rats (Mannaioni et al., 2003). 5-HI is a positive modulator of the 5-HT_3 receptor (Kooyman et al., 1993; van Hooft et al., 1997; Gunthorpe and Lummis, 1999) and α7 nicotinic acetylcholine receptors (Zwart et al., 2002; Selina Mok and Kew, 2006). However, the molecular mechanisms by which 5-HI affects the 5-HT_3 receptor are still poorly understood. Sites

Abbreviations: 5-HT_3, 5-hydroxytryptamine (serotonin) type 3; nACh, nicotinic acetylcholine; TM, transmembrane domain; 5-HI, 5-hydroxyindole; HEK cells, human embryonic kidney cells; WT, wild-type; DA, dopamine.

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involved in the binding and allosteric actions of 5-HI have proven elusive due to the complex effects on 5-HT_{3A} receptor-mediated responses and possible existence of more than one binding site. Previous studies suggested that 5-HI exhibits a combination of competitive and non-competitive actions at the 5-HT_3 receptor (Kooyman et al., 1994), suggesting that there are at least two binding sites for 5-HI. One site likely overlaps with the orthosteric agonist binding site, whereas the second site may be in another region of the protein.

It has been established that amino acid residues in the TM 2 domain and TM2–TM3 loop play critical roles in 5-HT_3 receptor gating (Filatov and White, 1995; Grosman et al., 2000; Grosman and Auerbach, 2000) and allosteric modulation (Boileau and Czajkowski, 1999; Hu and Lovinger, 2005; Jones-Davis et al., 2005) of the Cys-loop ligand-gated ion channels. To identify an amino acid residue(s) that participates in 5-HI modulation, we examined the roles of selected residues from TM2 domain of the 5-HT_{3A} receptor by site-directed mutagenesis and whole-cell patch-clamp recording.

2. Materials and methods

2.1. Mutagenesis

Point mutation of the mouse 5-HT_{3A} receptor (gift from Dr. D. Julius, San Francisco, CA) was accomplished using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The successful incorporation of mutations was verified by sequencing the clones using an ABI Prism 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). The cDNAs were then subcloned into the vector pCDNA3.1 (Invitrogen) for expression in human embryonic kidney (HEK) 293 cells (passage ≤ 12).

2.2. Cell culture and transient receptor expression

N1E-115 cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS), and maintained in a humidified incubator at 37 °C in 5% CO_2. HEK 293 cells (ATCC) were grown in minimum essential medium (MEM; Invitrogen) supplemented with 10% horse serum, and maintained in a humidified incubator at 37 °C in 5% CO_2. HEK 293 cells were transiently transfected with the wild-type or mutant 5-HT_{3A} 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_{3A} receptor subunit to permit selection of transfected cells under fluorescence optics. Each 35 mm dish was transfected with 3 μg of cDNA encoding the wild-type or mutant receptors along with 1 μg green fluorescent protein cDNA.

2.3. Whole-cell patch-clamp recording

Whole-cell patch-clamp recordings were performed in N1E-115 cells 1 day after cell passage, or HEK 293 cells 1–3 days after transfection. HEK 293 cells were re-plated on the day of the experiment to ensure that recordings were only made from single, isolated cells. Cells were continuously superfused with a solution containing 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES (pH was adjusted to 7.4 with NaOH and osmolarity adjusted to ~340 mosmol l^{-1} with sucrose). Pipettes were pulled from borosilicate glass (TW-150F; World Precision Instruments, Sarasota, FL) using a two-stage puller (Flaming–Brown P-97; Sutter Instruments, Novato, CA) and had resistances of 2–5 MΩ when filled with pipette solution containing 140 mM CsCl, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES (pH was adjusted to 7.2 with CsOH, and osmolarity adjusted to ~315 mosmol l^{-1} with sucrose). Membrane current was recorded in the whole-cell configuration using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) at 20–22 °C. Cells were held at −60 mV unless otherwise indicated. Data were acquired using pClamp9.0 Software (Axon). Currents were filtered at 2 kHz and digitized at 5–10 kHz.

Drugs were applied with a piezoelectric device (PZ-150M; EXFO Burleigh Products Group Inc., Victor, NY) through two-barrel theta glass tubing (TGC150, Warner Instruments, Hamden, CT) that had been pulled to a tip diameter of ~200 μm. The cell was placed in front of the stream of control solution. The piezoelectric device was driven by TTL pulses from the pClamp9.0 Software. Voltage applied to the piezoelectric device produced a rapid lateral displacement (~50 μm) of the theta tubing to move the interface between control and drug solutions. Solution exchange rates for open pipette and whole-cell recording were estimated using the potential change induced by switching from the control solution to a 140 mM *N*-methyl-D-glucamine (NMDG) test solution at 0 mV in the absence of agonist; and the current rising phase was fit using an exponential function. The solution exchange time constants were ~0.3 ms for an open pipette tip and ~1.6 ms for whole-cell recording.

2.4. Data analysis

Data analysis and curve fitting were performed with Origin7.0 (Microcal Software, Northampton, MA), pClamp9.0 (Axon), or GraphPad InStat3.0 (GraphPad Software Inc., San Diego, CA) Software. Concentration–response data for dopamine (DA) and 5-HI were fit using the Hill equation, $I/I_{\max\ 5\text{-HT}} = 1/[1 + (EC_{50}/[\text{Agonist}])^{n_h}]$, where I is the current amplitude activated by a given concentration of agonist ([Agonist]), $I_{\max\ 5\text{-HT}}$ is the current amplitude produced by a maximally efficacious concentration of 5-HT (30 μM), n_h is the Hill coefficient and EC_{50} is the concentration eliciting a half-maximal response.

Parameters of channel deactivation and desensitization were estimated by fitting appropriate current components using exponential functions of the general form $\sum A_n e^{(-t/\tau_n)} + A_s$, where A_n is the relative amplitude of the respective component, A_s is the steady-state current, n is the optimal number of exponential components, t is time and τ_n is the respective time constant. Curve fitting was achieved in Clampfit9.0 using the Levenberg–Marquardt algorithm. Additional components were accepted only if they significantly improved the fit, as determined by an F test performed using the analysis software.

Desensitization rates were derived from exponential fits to the current decay starting just after the current peak and extending to the end of agonist application, whereas deactivation rates were derived from exponential fits to the current decay after the removal of agonist following a 2-ms application. To facilitate direct comparison of desensitization or deactivation with different components, a weighted summation of time constants ($\sum a_n\tau_n$) was used, where a_n is the fractional contribution of the respective component, τ_n is the respective time constant, and n is the optimal number of exponential components. The extent of desensitization was measured as the percentage of current loss relative to peak current using the equation $[(I_{peak} - I_{10\ s})/I_{peak}] \times 100$, where I_{peak} is the peak current amplitude, $I_{10\ s}$ is the current amplitude at the end of 10 s application of 5-HT alone or 5-HT plus 5-HI.

In some experiments voltage ramps were applied to measure reversal potential. A ramp that changed the membrane potential between −80 and +60 mV with a slew rate of 0.5 mV ms^{-1} was applied during the peak of current activated by 3 μM 5-HT, 5 mM 5-HI, 5 mM 5-methoxyindole and 3-(2-hydroxyethyl)indole. Current activated by a voltage ramp in the absence of agonist was subtracted from the ramp-activated current in the presence of agonist prior to plotting and analyzing these data.

To facilitate comparison between different Cys-loop ligand-gated ion channels, a common TM2 domain numbering system is used (Miller, 1989). In this system, the amino acid residue at the putative cytoplasmic end of TM2 domain is assigned as position 1′ (Fig. 2A).

Data are presented as mean ± S.E.M. Statistical significance was determined with Student's t test or one-way analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

2.5. Kinetic simulations

Kinetic simulations of current measured in HEK 293 cells were generated using Berkeley-Madonna V8.3 Software (Macey and Oster, Berkeley, CA) and the Runge–Kutta 4 integration algorithm. Channel states and most rate constant estimates were as in the allosteric model developed by Solt et al. (2007) except where noted. For simulation of continuous 5-HT application, 10 s of simulated current was generated in the presence of 30 μM 5-HT with all channels initially set in the unliganded, closed state (R). For simulation of "deactivation", 20 s of simulated current was generated with all channels initially set in the triple-liganded, open state (A_3O) and agonist concentration set to 0. The figure showing simulated current was generated in Prism Software using exported simulated traces.

3. Results

3.1. Positive modulation of 5-HT_3 receptor-mediated current by 5-HI

To study the effects of 5-HI, we examined whole-cell currents activated by 5-HT in N1E-115 neuroblastoma cells expressing the native mouse 5-HT_3 receptor (Neijt et al., 1988; Hope et al., 1993), and subsequently in HEK 293 cells transiently expressing the recombinant mouse 5-HT_{3A} receptor. Inward current with a rapid onset was observed upon application of a maximally efficacious concentration of 5-HT (30 μM) for 10 s to either N1E-115 cells or transfected HEK 293 cells (Fig. 1A, left). Co-application of 5 mM 5-HI with 5-HT enhanced the current mediated by the 5-HT_{3A} receptor in both cell lines (Fig. 1A, right). The average peak current amplitude activated by 5-HT in the presence of 5-HI was ~120% of that in the absence of 5-HI (N1E-115: 125 ± 6%; HEK 293: 119 ± 8%), which is