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Importance of recognition loops B and D in the activation of human 5-HT$_3$ receptors by 5-HT and meta-chlorophenylbiguanide

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ABSTRACT

The 5-HT$_3$ receptor is a cation selective member of the pentameric Cys-loop ligand-gated ion channels. While five subunits are known to exist, only two receptor subtypes have been significantly characterized: the homomeric receptor consisting of five A subunits and the heteromeric receptor containing both A and B subunits. The agonist recognition and activation of these receptors is orchestrated by six recognition loops three, A–C, on the principal subunit, and three, D–F, on the complementary subunit. In this study we have focused on the B loop of the principal subunit and loop D of the complementary subunit where aligned amino acids differ between the two subunits. A mutational analysis has been carried out using both 5-HT and m-chlorophenylbiguanide (mCPBG) to characterize receptor activation in the mutant receptors using two-electrode voltage clamp in *Xenopus* oocytes. The results show that the B loop W178I mutation of the 5-HT3A subunit markedly reduces the efficacy of mCPBG in both the homomeric and heteromeric receptors, while activation by 5-HT remains intact. Replacement of the D loop amino acid triplet RQY of the 5-HT3A subunit, with the aligned residues from the 5-HT3B subunit, QEV, converts 5-HT to a weak partial agonist in both the homomer and heteromer, but does not compromise activation by mCPBG. Exchange of the RQY triplet for the 5-HT3B subunit homologue, QEV, increases the Hill coefficient and decreases the EC$_{50}$ of this mutant when expressed with the wild type 5-HT3A subunit.

1. Introduction

The 5-HT$_3$ receptor (5-HT$_3$R) is a member of the Cys-loop family of ligand-gated ion channels, and, like the archetypal member of the family, the nicotinic acetylcholine receptor (nAChR), it is cation selective. The GABA$_A$ and strychnine sensitive glycine receptors represent the anion selective receptors of the family. Five genes have been associated with the 5-HT$_3$R and are designated A–E, but only two receptor subtypes have been significantly characterized: the homomeric 5-HT$_3$A and the heteromeric 5-HT$_3$AB receptors. The 5-HT$_3$R exhibits pentameric pseudosymmetry and fits into a cylinder with a diameter of 8 nm and a long axis of 11 nm, perpendicular to the cell membrane (Boess et al., 1995). The subunits exhibit around 20% homology to those of the nAChR from *Torpedo marmorata*, the structure of which has been resolved to 4Å using cryo-electron microscopy (Unwin, 2005). This receptor family exhibits three domains: an extracellular domain (ECD), a transmembrane domain (TMD) and an intracellular domain (ICD). The ECD forms a 3 nm diameter ion vestibule and is responsible for agonist recognition. The TMD contains four helices from each subunit, the second, TM2, forms the lining of the ion pore that gates ion flow. The ICD is incompletely resolved but exhibits a single membrane associated (MA) helix which has been shown to restrict channel conductance of the homomeric 5-HT$_3$AR (Kelley et al., 2003).

Agonist recognition within this receptor family appears to be delineated by six non-contiguous loops at the subunit interfaces, three, A–C, associated with the principal subunit and three, D–F with the complementary subunit. Much of the information about the importance of individual amino acids within these loops of this particular member of the family has been accrued from mutagenic studies carried out on the homomeric 5-HT$_3$AR (see Barnes et al., 2009; Lummis, 2012 for amplification), although it is the heteromeric 5-HT$_3$AB receptor that exhibits characteristics which more closely reflect those of neuronal receptors (Fletcher and Barnes, 1998; Davies et al., 1999). There is little evidence for the

Abbreviations: 5-HT, 5-hydroxytryptamine; mCPBG, meta-chlorophenylbiguanide; 5-HT$_3$AR, 5-hydroxytryptamine type 3A receptor; 5-HT$_3$ABR, 5-hydroxytryptamine type 3AB receptor; nAChR, nicotinic acetylcholine receptor; wt, wild type.

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[1] This work is dedicated to the memory of Susan M.J. Dunn (1954–2011).

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differentiation of the two receptor subtypes by competitive antagonists (Brady et al., 2001) although it has recently been reported that this can be achieved by a novel antagonist, exhibiting higher affinity for the homomer than the heteromer (Thompson et al., 2012). The two receptor subtypes exhibit distinct concentration–response curves to the natural agonist in human where the presence of the B subunit increases the relative efficacy of mCPBG and 1-phenylbiguanide (1-PBG) compared to the natural agonist 5-HT (Dubin et al., 1999), compatible with the view that agonist activation in the homomeric and heteromeric receptors is distinct.

In this study we have explored further the distinct receptor activation characteristics of two agonists, 5-HT and mCPBG, focussing on selected amino acids from recognition loops B and D, found to be important in agonist activation of the homomeric receptor by 5-HT. Loop B of the A subunit contains a tryptophan residue which forms an important cation-π interaction with the aliphatic amine of the natural agonist 5-HT in the homomeric receptor (Beene et al., 2002), while the aligned position of the B subunit is occupied by isoleucine, which is unable to support such an interaction. Comparison of the D loop between the A and B subunits shows that they contain a distinct triplet of amino acids, of particular interest as this loop appears to be important in activation, but not recognition, in nAChR (Akk, 2002). We have replaced these residues in the A subunit by those found in homologous positions of the human B subunit; the converse experiments have also been carried out in which residues in the B subunit have been replaced by those in the homologous positions of the A subunit. The A subunit mutants have been expressed alone or together with the wild type (wt) B subunit; the B subunit mutants have been expressed with the wt A subunits. Expression was carried out in *Xenopus* oocytes and the receptors functionally characterized using two-electrode voltage clamp.

2. Experimental procedures

2.1. Materials

All drugs were purchased from Sigma–Aldrich (St. Louis, MO, USA) and were made as stock solutions (3–10 mM) in sterile water or frog Ringer's buffer (110 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$ and 5 mM HEPES, pH 7.4). mCPBG was heated and then sonicated to make 3 mM solutions. Restriction enzymes and cRNA transcript preparation materials were purchased from Invitrogen (Burlington, ON, Canada), Promega (Madison, WI, USA), or New England Biolabs (Pickering, ON, Canada). *Pfu* Turbo DNA polymerase, for site directed mutagenesis was purchased from Stratagene (La Jolla, CA, USA). Custom primers were prepared by IDT (Coralville, IA, USA).

2.2. Site-directed mutagenesis

Mutagenesis was performed as previously described (Derry et al., 2004). Briefly, the QuickChange protocol (Stratagene, La Jolla, CA) was used to introduce the B loop I176W and separately the D loop triplet QEV-85, 86, 87-RQY into the wild-type human 5-HT3B subunit cDNA sequence. The homologous mutations for the 5-HT3A subunit B loop W178I, and the D loop RQY-87, 88, 89-QEV were also constructed (full alignments can be found in Supplementary Fig. 1). The individual subunit cDNA sequences, 5-HT3A (accession number P40698) and 5-HT3B (accession number O95264), had been previously subcloned into pcDNA3.1(+) (Invitrogen, San Diego, CA) and these plasmids were used as templates in polymerase chain reaction-mediated (PCR) mutagenesis protocols. The mutants were screened by restriction endonuclease digestion and all mutations were confirmed by DNA sequencing. *In vitro* transcription of cRNA was performed using standard protocols (Invitrogen).

2.3. Expression of receptors in Xenopus oocytes

Stage V–VI *Xenopus laevis* oocytes were isolated and prepared as previously described (Smith et al., 2004). Oocytes were microinjected with 50 nL total of 1 μg/μL wild-type or mutant 5-HT3A and mutant or wild-type 5-HT3B subunit cRNA in a 1:1 ratio. Injected oocytes were incubated in ND96 buffer (96 mM NaCl, 1.8 mM $CaCl_2$, 2 mM KCl and 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4 with NaOH) containing 50 μg/mL gentamicin (GIBCO, Grand Island, NY, USA) in 96-well plates at 14 °C for 2–7 days prior to functional analysis.

2.4. Fluorescent visualization of 5-HT3B subunit mutants in Xenopus oocytes

Five days after mRNA injection, oocytes were fixed in Z-fix for 15 min at 4 °C. Oocytes were rinsed with PBS containing 50 mM NH_4Cl then blocked for 30 min with 2% bovine serum albumin (BSA) in PBS. Rabbit anti-5-HT3B (Sigma–Aldrich Canada, Oakville, Canada Cat.AV35186) directed to the N-terminal region of the protein was diluted in blocking buffer and applied to oocytes overnight. Oocytes were washed in PBS for 30 min, followed by incubation for 1 h with a chicken anti-rabbit Alexa 488 secondary antibody (Molecular probes, Eugene, OR) diluted in blocking buffer. Oocytes were rinsed in PBS for 30 min and imaged with a 20× objective on a spinning disk confocal microscope (Perkin Elmer UltraView ERS6) using GFP laser (488 nm). The acquisition was performed with a Hamamatsu digital camera (C9100-50 EM CCD) and analysed with Volocity 5.5.1 software.

2.5. Electrophysiological recordings

Oocytes were continuously bathed in frog Ringer's buffer by gravity flow (~5 mL/min) in a custom-made recording chamber. Agonist-induced currents were measured by standard two-electrode voltage clamp techniques using a GeneClamp 500B amplifier (Axon Instruments Inc., Foster City, CA, USA) at a holding potential of −60 mV. Electrodes were filled with 3 M KCl and had a resistance between 0.3 and 1.5 MΩ in Frog Ringer's solution. Oocytes were only used for experiments when current responses were stable (±10%) between successive applications of agonist. All stock solutions of drugs were diluted in perfusion (frog Ringer's) buffer.

To measure the effects of 5-HT and mCPBG, the agonist was applied via gravity perfusion for 10–30 s. This was followed by an 8–12 min washout to ensure complete recovery from desensitization.

2.6. Data analysis

Concentration–effect curves for agonist activation were analysed by non-linear regression techniques using GraphPad Prism 5.00 software (San Diego, CA, USA) and the following equation:

$$I = \frac{I_{max} * [L]^{nH}}{EC_{50}^{nH} + [L]^{nH}}$$

where I is the amplitude of agonist-evoked current for a given concentration [L], I_{max} is the maximum amplitude of current, EC_{50} is the agonist concentration that evokes half maximal receptor activation, and nH is the Hill coefficient. EC_{50}, which is log normally