Mutational analysis of the mouse 5-HT_7 receptor: importance of the third intracellular loop for receptor–G-protein interaction

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Abstract The mouse serotonin (5-HT) receptor subtype, 5-HT_7, belongs to the family of seven transmembrane G-protein-coupled receptors. To identify the structural basis for the coupling of 5-HT_7 receptor to $G\alpha_S$ we constructed a number of receptor mutants in which amino acid residues were either substituted or deleted from the second and third intracellular loops. Wild-type and mutant 5-HT_7 receptors were expressed in insect cells using the baculovirus vectors. Two mutant receptor species, 5-HT_7(E325G) and 5-HT_7(K327S), demonstrated markedly impaired abilities to stimulate adenylyl cyclase. The results suggest the importance of the C-terminal region of the third intracellular loop in receptor–G-protein interaction and that specific charged residues, E325 and K327, may play a critical role in this interaction.

Key words: 5-HT_7 receptor; G-protein; Mutagenesis; Baculovirus expression

1. Introduction

A large number of receptors belong to the family of seven transmembrane (7TM) receptors which are coupled to their intracellular effectors via guanine nucleotide binding proteins (G-proteins). These receptors are believed to share a common membrane topology consisting of seven α-helical membrane-spanning segments connected by alternating extracellular and intracellular loops. The best characterised 7TM domain G-protein-coupled receptor is the β-adrenergic receptor [1]. Mutational analysis of this receptor and other G-protein-coupled receptors has shown that a ligand binding pocket is formed by the 7TM spanning domains, while the amino acid sequences within the intracellular loops are involved in functional coupling of the receptor to G-proteins [1,2]. The 13 different mammalian receptors which bind the biogenic amine serotonin (5-hydroxytryptamine, 5-HT), also belong to the superfamily of 7TM domain G-protein-coupled receptors, with the exception of the 5-HT_3 receptor which forms a serotonin gated ion channel [3]. Four 5-HT_7 receptor subtypes have been identified by cloning from the following mammalian species; mouse [4], rat [5–8], human [9] and guinea pig [10]. When expressed in cultured mammalian cell lines these receptors were found to stimulate adenylyl cyclase via the stimulatory G-protein, $G\alpha_S$. In this study we were interested in defining which regions of the mouse 5-HT_7 receptor were critical for the interaction with $G\alpha_S$. To address this question we used the wealth of information available on the β_2 adrenergic receptor (β_2-AR) [1,2]. Studies on this receptor have shown that several amino acids in the second intracellular loop, and both the amino (N)-terminal and carboxyl (C)-terminal regions of the third intracellular loop are required for the interaction with $G\alpha_S$ [1,2]. We generated six mutant 5-HT_7 receptors which contained mutations within the second intracellular loop and in the N- and C-terminal portions of the third intracellular loop of this receptor. Mutant 5-HT_7 receptors were expressed in *Spodoptera frugiperda* (Sf9) cells using baculovirus expression vectors, and were initially examined for their ability to bind both antagonist and agonist ligands. Two mutant receptors, 5-HT_7 (E325G) and 5-HT_7 (K327S) which not only had comparable expression levels to that of the wild-type receptor but also displayed similar affinities for agonist and antagonist ligands were identified. More significantly, these mutant receptors exhibited a marked impairment in ability to stimulate adenylyl cyclase when compared to the wild-type receptor. The data presented here suggests a functional role for the C-terminal portion of the third intracellular loop of the mouse 5-HT_7 in receptor coupling to $G\alpha_s$ and that this interaction may involve charged residues present in this domain.

2. Materials and methods

2.1. Expression vector construction and mutagenesis procedures

The plasmid p514/5-HT_7 containing a 1539 bp cDNA fragment encoding the wild-type mouse 5-HT_7 receptor was subjected to the polymerase chain reaction (PCR) amplification technique using oligonucleotide primers designed to amplify the coding region of the 5-HT7 cDNA. PCR amplifications were performed using the high-fidelity *Pfu* DNA polymerase (Stratagene) and the resultant 1355 bp reaction product was cleaved with *Eco*RI and cloned into the baculovirus transfer vector pAcAL3 [11]. The authenticity of the DNA fragment was confirmed by double-stranded dideoxynuleotide sequencing.

Mutant 5-HT_7 receptors were generated by overlap extension PCR essentially as described by [12] except that reactions were performed with *Pfu* DNA polymerase. The correct identity of each DNA sequence was confirmed by double-stranded dideoxynuleotide sequencing. To construct the deletion mutant 5-HT_7(Δ263–275), PCR products were obtained using the following sets primers: (a) 5′-CGCGGATCCACGATGATGGACGTT-3′/5′-GTACATGAACAG-CATGAC-3′ (b) 5′-CACAAGTTCTCAGGCTTC-3′/5′-TTTGAAT-TCATCATGTATCATGACCTT-3′. Products (a) and (b) were restricted with the enzymes *Bam*HI and *Eco*RI, respectively, and a three-component ligation was performed consisting of products (a) and (b) and the plasmid vector. Following, double-stranded DNA sequencing to verify the location of the junction site and to ensure that additional mutations had not been introduced, the DNA fragment was subsequently ligated into the baculovirus transfer vector pAcAL3 [11].

2.2. Production of recombinant baculoviruses

All procedures for cell culture, identification and amplification of recombinant viruses were as detailed [11]. Recombinant viruses were produced by co-transfection of Sf21 cells with linearized BacPAK6 DNA [13] and the appropriate transfer vector, using lipofectin (Gibco Life Technologies). For routine experiments, Sf9 cells cultured in serum-free media (Sf900II, Gibco Life Technologies) were used.

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2.3. Receptor binding assays

Virus-infected Sf9 cells were harvested at 48 hpi and used to prepare membranes as previously described [14]. For saturation experiments, membrane suspensions (20–30 μg protein) were incubated with various concentrations of [^{3}H]5-HT ranging from 0.16 to 30 nM in a total volume of 0.25 ml. Competition binding assays with agonist or antagonist were conducted using [^{3}H]5-HT (3.0 nM) and [^{125}I]LSD (20 pM) with concentrations of unlabelled ligands which varied from 1 μM to 0.1 pM. All reactions were performed in triplicate and the binding data were analyzed by non-linear least-squares regression using the computer program LIGAND [15].

2.4. Adenylyl cylase assays

Cell membranes were prepared from virus-infected Sf9 cells at 48 hpi as described [14] and used to determine adenylyl cyclase activities as detailed by [16]. Enzyme reactions were initiated by the addition of prepared membranes (30–40 μg protein) to reaction mixes in the absence or presence of various concentrations of 5-HT. Reactions were performed in triplicate and data were calculated as picomoles of cAMP produced per minute per milligram of membrane protein and analysed using non-linear least-squares regression with the aid of the computer program GraphPad Prism (San Diego, CA).

3. Results and discussion

Using the plasmid p514/5-HT$_7$ as template DNA, a 1355 bp fragment encoding the mouse 5-HT$_7$ receptor was amplified by PCR and cloned into the baculovirus transfer vector pAcAL3. This step was essential in order to remove a potential ATG translation start site from the 5′ non-coding region of the 5-HT$_7$ cDNA. A recombinant baculovirus (Ac5HT$_7$) was subsequently constructed in which the 1355 bp DNA fragment was placed under control of the highly efficient polyhedrin promoter. To assess the levels of receptor expression, saturation experiments were performed using membranes prepared from Ac5HT$_7$-infected Sf9 cells harvested at 48 hpi. Fig. 1A shows that the binding of [^{3}H]5-HT was saturable and non-linear regression analysis of this binding data revealed the existence of a single class of receptor binding site with an affinity constant (K_d) of 3.93 nM (±0.46, $n=4$), and $B_{max}=24.3$ (±5.2) pmol/mg of membrane protein. A major characteristic of the 5-HT$_7$ receptor subtype is that it displays high affinity for the 5-HT$_1$ agonist, 5-CT. In this study, binding of [^{3}H]5-HT was shown to be displaced with high affinity by 5-CT (IC_{50} 1.3±0.04 nM, Fig. 1B), and in addition by the non-selective 5-HT receptor antagonist methysergide (IC_{50} 18.0±1.0 nM, Fig. 1B). As expected, these data were in agreement with previous studies [4] and indicated that the mouse 5-HT$_7$ receptor molecule synthesized in insect cells exhibited ligand binding properties similar to those observed in the mammalian COS-7 cell expression system. However, levels of 5-HT$_7$ receptor expression in insect cells were observed to be higher (≈3.5-fold) than previously reported [4] thus highlighting one of the advantages of the baculovirus expression system. Further characterisation of the 5-HT$_7$ receptor involved confirmation that the receptor would stimulate adenylyl cyclase via the stimulatory G-protein, Gα$_S$. As shown in Fig. 3, adenylyl cyclase activity was found to increase in a dose-dependent manner (EC_{50} 48.0±1.2 nM, Fig. 3) in membranes prepared from Ac5HT$_7$-infected Sf9 cells and incubated with various concentrations of 5-HT. This stimulation was also inhibited in a dose-dependent manner by methiothepin (75.0±0.8 nM, data not shown).

Once functional 5-HT$_7$ receptor synthesis in Sf9 insect cells had been established, we undertook a mutagenic study to define receptor domains responsible for the productive coupling to the stimulatory G-protein (Gα$_S$). Various studies on the β$_2$-AR and other receptors that couple to Gα$_S$ [1,2] suggest that binding of the agonist induces a conformational change in the receptor molecule which allows the intracellular portions of the receptor to interact with the G-protein. This evidence has implicated the second and third intracellular loops as well as the C-terminal tail of the β-AR as all playing some role in receptor–G-protein coupling [2]. The deletion of small segments of amino acids from the N- and C-terminal portions of the third intracellular loops demonstrated the contribution of these domains to β$_2$-AR-mediated activation of adenylyl cyclase [2].

Mutant mouse 5-HT$_7$ receptors were generated in which amino acid substitutions or deletions were made in N- and C-terminal portions of the third intracellular loop as well as the second intracellular loop. In this study we focused on the

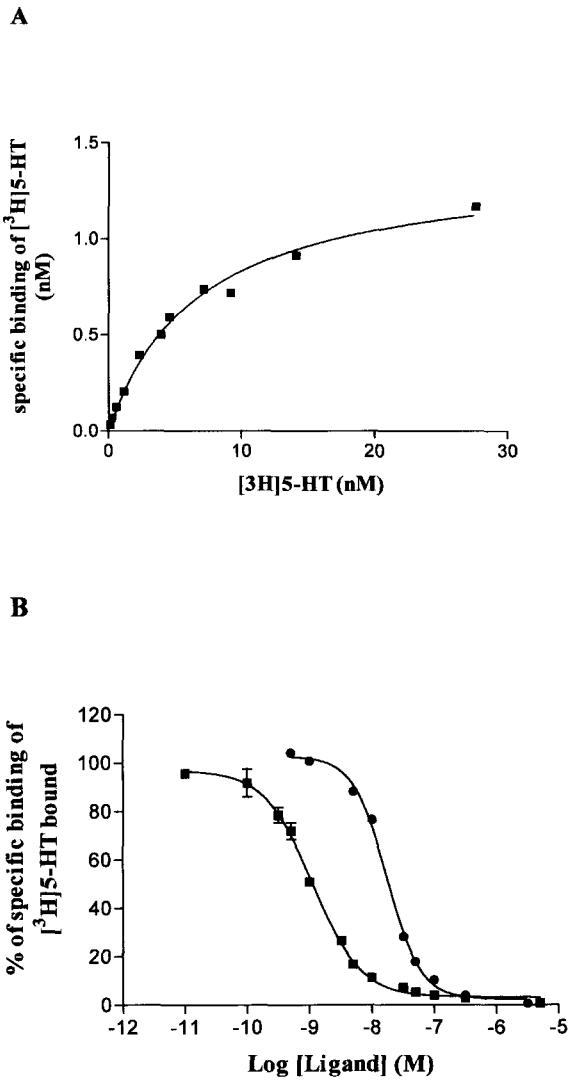

Fig. 1. A: Saturation binding curve of [^{3}H]5-HT to membranes prepared from Ac5-HT$_7$ (WT) infected Sf9 cells. Data are the mean of triplicate determinations from a single experiment typical of four independent experiments. B: Displacement of [^{3}H]5-HT binding to membranes prepared from Ac5-HT$_7$ (WT) infected Sf9 cells. The binding of 3 nM [^{3}H]5-HT to membranes was performed in the presence of 5-CT (■) or methiothepin (●). Data are representative of two independent experiments with each point measured in triplicate.

role of charged amino acid residues found in these receptor domains and substituted these amino acids for neutral alanine or serine residues in order to determine if the high proportion of charged amino acids were required for productive coupling to $G\alpha_S$. Previous studies by [17] suggested that for the hamster β2-AR charged amino acids in the N-terminal region of the third-intracellular loop were less critical for receptor–G-protein interaction than hydrophobic residues. It was thus of interest to establish the relevance of this observation in relation to the mouse 5-HT_7 receptor. In all cases the DNAs encoding the mutant mouse 5-HT_7 receptors were verified by double-stranded DNA sequencing, to ensure both the presence of the mutation but also to confirm that additional mutations had not been produced. Recombinant baculoviruses encoding the various mutant receptor proteins were produced and subsequently expressed in Sf9 insect cells. Receptors with mutations in the N-terminal portion of the third intracellular loop were designated 5-HT_7(K267A), 5-HT_7-(K267A,R270A,K271A) and 5-HT_7(Δ263–275). Those receptors with mutations the C-terminus of this loop were referred to as 5-HT_7(E325G) and 5-HT_7(K327S). While a receptor

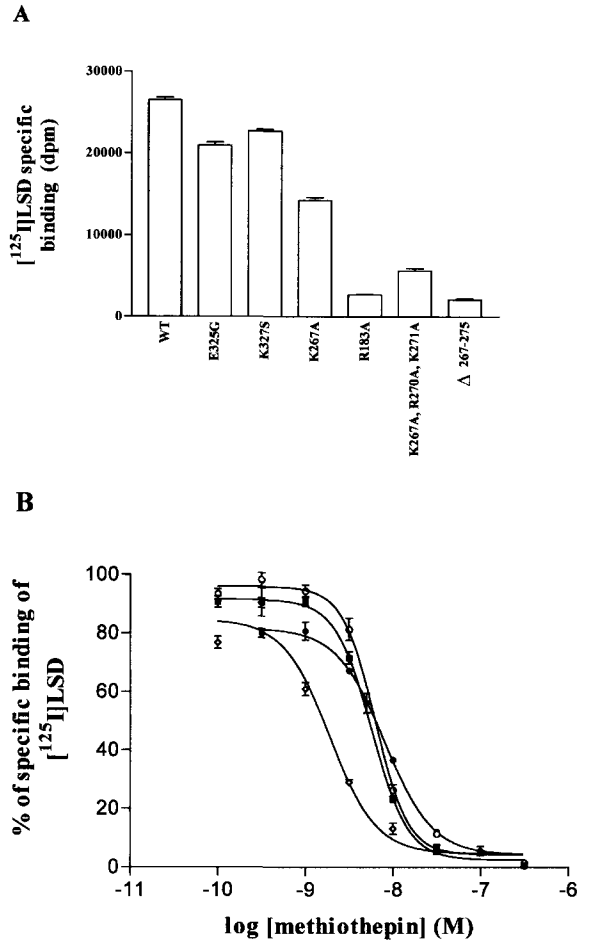

Fig. 2. A: Specific binding of 0.1 nM [^{125}I]LSD to wild-type and mutant 5-HT_7 receptors expressed in Sf9 cells (data are mean ± SEM, $n = 3$). B: Competition binding of wild-type and mutant receptors. Competition binding of methiothepin with [^{125}I]LSD (20 pM) to membranes prepared from Sf9 cells expressing 5-HT_7(WT) (○), 5-HT_7(K327S) (■), 5-HT_7(E325G) (●) and 5-HT_7-(R183A) (◇) receptors.

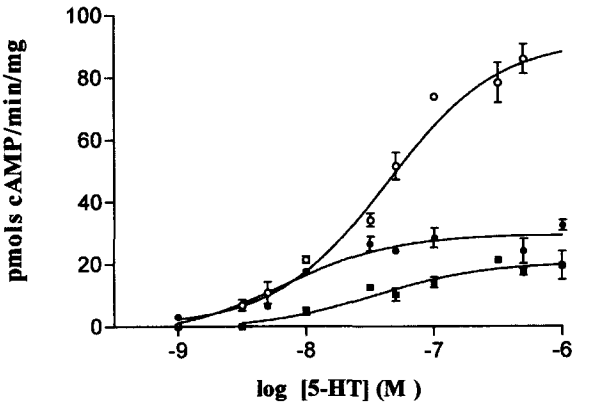

Fig. 3. Stimulation by 5-HT of adenylyl cyclase in Sf9 cells expressing wild-type and mutant 5-HT_7 receptors. Adenylyl cyclase activity was measured in the presence of 5-HT in membranes derived from Sf9 cells infected with the recombinant baculoviruses Ac5HT_7(WT) (○), Ac5HT_7 (E325G) (●) and Ac5HT_7(K327S) (■). Data are the mean ± SEM of 2–3 independent experiments with each point determined in triplicate.

with a single point mutation in the second intracellular loop was designated 5-HT_7(R183A).

Ligand binding experiments using [^{125}I]LSD were initially undertaken to evaluate the effects of the mutations on the overall levels of receptor expression in insect cells (Fig. 2A). The deletion of 13 amino acids in the mutant 5HT_7(Δ263–275) caused a reduction in [^{125}I]LSD binding capability presumably due to structural constraints introduced by this deletion. Similarly, the receptor mutant 5-HT_7(K267A), and the triple mutant 5-HT_7(K267A,R270A,K271A), showed marked reductions in ability to support ligand binding also suggesting incorrect folding or membrane insertion of the receptors in the plasma membrane. For this reason the signalling properties of these mutant receptors were not studied. Of interest is the mutant receptor 5-HT_7(R183A) in which the invariant arginine (R183) residue in the DRY sequence motif was substituted for alanine. This motif which is found in a large number G-protein-coupled receptors is thought to be important for receptor–G-protein coupling [11,12]. Substitution of this invariant arginine has previously been reported to result in loss of coupling to $G\alpha_S$ in the case of the VP2 vasopressin receptor [18] or $G\alpha_q$ for the α_{1B}-adrenergic receptor [19]. In either case no concomitant loss in ligand binding was observed. The mutant 5-HT_7(R183A) was further characterized by an apparent increase in affinity for the antagonist methiothepin (IC_{50} 1.9 ± 0.06 nM, Fig. 2B) when compared to the wild-type receptor (IC_{50} 6.2 ± 0.03 nM). It would therefore appear that R183 is required for maintaining the structural integrity the mouse 5-HT_7 receptor.

On the strength that the receptor mutants 5-HT_7(E325G) and 5-HT_7(K327S) appeared to be correctly processed and expressed (Fig. 2A), these receptor species were further characterized. When the 5-HT receptor antagonist methiothepin was used to compete for [^{125}I]LSD binding, the 5-HT_7(E325G) and 5-HT_7(K327S) receptors displayed similar affinities (IC_{50} of 8.0 ± 0.03 and 5.7 nM, respectively) to that of the wild-type receptor (IC_{50} 6.2 ± 0.03 nM). In addition, saturation experiments using the radiolabeled agonist [^{3}H]5-HT, revealed no significant changes ($P < 0.05$) in affinity for this ligand. The apparent K_d values were as follows; 5-HT_7(E325G): 3.9 ± 0.6 nM ($n = 3$), 5-HT_7(K327S),

K_d 5.3 ± 0.4 nM ($n = 3$) receptors. Furthermore, no significant changes in B_{max} values were obtained.

Given that there were no apparent changes in the pharmacological binding properties of the 5-HT_7(E325G) and 5-HT_7(K327A) the abilities of these mutants to modulate adenylyl cyclase activity in the presence of 5-HT were investigated. As shown in Fig. 3, both mutant receptors exhibited significantly impaired (> 50%) abilities to stimulate adenylyl cyclase (Fig. 3) implying a critical role of residues E325 and K327 in receptor–G-protein interaction. The EC_{50} for the mutant receptor 5-HT_7(E325G) was 10.0 ± 0.2 nM while that for the mutant 5-HT_7(K327A) receptor could not be reliably determined due to the lack of a suitable inflection point for the 5-HT-induced adenylyl cyclase activity.

Our results have identified two amino acids residues which are important for coupling of the mouse 5-HT_7 receptor to $G\alpha_S$ proteins. Substitution of E325 for G or K327 for S in the receptor mutants 5-HT_7(E325G) and 5-HT_7(K327S) respectively, impaired receptor–G-protein coupling as inferred from the reduction in ability to stimulate adenylyl cyclase. In either case these amino acid substitutions were not associated with any changes in the agonist binding properties of the receptor. The presented data are in agreement with the view that the C-terminal region of the third intracellular loop of G-protein-coupled receptors contains some of the information required for effective coupling. The specificity of these mutations and the fact that charged residues E325 and K327 were substituted does signify that productive interaction of the mouse 5-HT_7 receptor with $G\alpha_S$ proteins may perhaps be governed by electrostatic interactions. However, the inability to completely abolish the 5-HT stimulated activation of adenylyl cyclase indicates the requirement of additional residues or domains in this interaction. Previous studies [20] have implied that the charged amino acid residues found in either the N- or C-terminal regions are unlikely to be the determinants of efficient receptor–G-protein coupling. In this respect our findings differ from these observations and could reflect a specific feature of 5-HT receptors that couple to $G\alpha_S$.

Finally, in addition to establishing if substitution of both residues E325 and K327 does abolish cAMP production we are currently investigating the role of other charged amino acids in C-terminal region in the third intracellular loop of the mouse 5-HT_7 receptor. Although, the site-specific mutations that were made in the N-terminal region of the third intracellular loop and the second intracellular loop resulted in an apparent loss of receptor function the present data cannot exclude the role of these domains in receptor–G-protein interaction. Taken together, however, our results demonstrate the requirement for maintenance of the structural integrity of all intracellular domains for productive coupling of the 5-HT_7 receptor with $G\alpha_S$.

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