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Bacterially expressed human serotonin receptor 3A is functionally reconstituted in proteoliposomes

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Abstract

Human serotonin receptor 3A (5-HT_{3A}) is a ligand-gated ion channel regulated by serotonin. A fusion protein (P9-5-HT_{3A}) of 5-HT_{3A} with the P9 protein, a major envelope protein of bacteriophage phi6, was highly expressed in the membrane fraction of *Escherichia coli*, and the expressed protein was purified to homogeneity using an affinity chromatography. P9-5-HT_{3A} was observed as mixed oligomers in detergents. The purified P9-5-HT_{3A} was efficiently reconstituted into proteoliposomes, and the serotonin-dependent ion-channel activity of P9-5-HT_{3A} was observed by measuring the increased fluorescence of Fluo-3 attributed to the formation of a complex with the Ca^{2+} ions released from the proteoliposomes. Alanine substitution for Trp178 of 5-HT_{3A} abolished the serotonin-dependent ion-channel activity, confirming the importance of Trp178 as a ligand-binding site. Furthermore, the ion-channel activity of the reconstituted P9-5-HT_{3A} was effectively blocked by treatment with ondansetron, an antagonist of 5-HT_{3A}. The bacterial expression system of human 5-HT_{3A} and the proteoliposomes reconstituted with 5-HT_{3A} would provide biophysical and structural analyses of 5-HT_{3A}.

Keywords

Serotonin; Receptor; 5-HT_{3A}; Overexpression; Purification; Reconstitution

Introduction

Neurotransmitter 5-hydroxytryptamine (5-HT) or serotonin receptors are grouped into seven families. Unlike other serotonin receptor families, which are G-protein coupled receptors, 5-HT_3 is a ligand-gated ion channel [1]. Among the five subunits of 5-HT_3 (5-$HT_{3A–E}$), the 5-HT_{3A} subunit has been extensively characterized both genetically and biochemically. The 5-

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HT_{3A} subunit shows sequence homology to the nicotinic/GABA receptor gene super family [2], and it is most closely related to the nicotinic acetylcholine receptor (nAChR) with 30% amino acid homology [3]. The 5-HT_{3A} subunit consists of an N-terminus extracellular domain, four transmembrane helical domains (M1–M4), and a relatively large cytoplasmic region between the M3 and M4 helixes. The 5-HT_3 receptor consists of a pentamer of 5-HT_3 subunits, and the 5-HT_{3A} subunit is essential for assembly with other subtype subunits to form functional channels [2,4]. When only the 5-HT_{3A} subunit is expressed in oocytes, it shows similar physiological characteristics to native 5-HT_3 [2], suggesting that the 5-HT_{3A} subunit could form an active homopentamer. Although a crystal structure of the 5-HT_{3A} subunit has not been reported, electron microscopic analysis [5], molecular modeling of the 5-HT_{3A} subunit based on a 4 Å resolution model of nAChR [6,7], and the crystal structure of the extracellular region of nAChR [8] all suggest that 5-HT_{3A} subunit has a pentameric quaternary structure, and the ligand-binding extracellular region consists predominantly of β-sheets [9]. Furthermore, it was found that the loop between the M2 and M3 helices may have an important role in channel opening or receptor desensitization [10,11]. However, structural analysis of the open and closed states of the 5-HT_{3A} subunit along with the principle behind this conformational change must be elucidated to understand the mechanism of ligand-gated ion channels for the purpose of drug development.

Structural and functional analyses of cell surface receptors require high-level expression in a heterologous system. Mammalian 5-HT_3 is expressed at a low level in the central nervous system [12]. However, 5-HT_3 is expressed at a substantially high level in mouse neuroblastoma cells, from which it can be purified and functionally reconstituted [13]. Nonetheless, structural and biochemical characterizations require a more convenient expression and purification system. The expression of membrane proteins in bacteria has been accomplished using specialized fusion partners such as Mistic [14], green fluorescent protein (GFP) [15], or maltose-binding protein and thioredoxin [16]. Recently, we had successively expressed human endothelin receptor type A (ET_A), a member of the G-protein coupled receptors, in *Escherichia coli* using the P9 protein of bacteriophage phi6 as a fusion partner of ET_A [17]. Most of the expressed P9-ET_A was located in the membrane fraction, and it could be purified to homogeneity with high yield, suggesting that the P9 protein could be used as a fusion partner to assist in high-level expressions of other membrane proteins such as 5-HT_{3A} in the membrane fraction of *E. coli*.

In this report, we have shown that the P9 protein was also effective in expressing human 5-HT_{3A} at a high level in the membrane fraction of *E. coli*. The expressed P9-5-HT_{3A} fusion protein was purified to homogeneity using a single-step affinity chromatography, with high yield. Furthermore, the purified P9-5-HT_{3A} was reconstituted into proteoliposomes, which showed the serotonin-dependent release of encapsulated Ca^{2+} ions.

Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), and 1,2-dioleoyl-*sn*-glycero-3-{[*N*-(5-amino-1-carboxylpentyl)-iminodiacetic acid]succinyl} (nickel salt) (DOGS-NTA-Ni) were purchased from Avanti Polar Lipids, Inc. (USA). Fluo-3 was obtained from Invitrogen (USA).

Serotonin, ondansetron, and *N*-lauroylsarcosine (Sarkosyl) were from Sigma (USA). Ni-NTA agarose resin was purchased from Qiagen (Germany). Bio-Beads (20–50 mesh) were from Bio-Rad (USA). All other chemicals used were of reagent grade.

Expression and purification of recombinant P9-5-HT_{3A}

The expression vector of recombinant 5-HT_{3A} (pP9-5-HT_{3A}) was prepared by inserting the coding region of human 5-HT_{3A} (amino acids 2-478; GenBank ID: CAA06442) at the C-terminus of the P9 protein from the *Pseudomonas* phage phi6 (GenBank ID: ABB69810.1) with a C-terminal His6 tag (amino acids: EASHHHHHH) (Fig. 1A). This sequence was inserted at the *Nde*I and *Hin*dIII sites of pRSETA (Invitrogen, USA) to generate the expression vector of P9-5-HT_{3A}. The expression vector for the W178A substitution mutant of P9-5-HT_{3A} [pP9-5-HT_{3A}(W178A)] was prepared by site-directed mutagenesis using pP9-5-HT_{3A} as the template DNA. The nucleotide sequences of the prepared expression vectors were confirmed by DNA sequencing. These vectors were transformed into the Rosetta2 (DE3) strain of *E. coli* and grown in Luria–Bertani medium containing 100 μg/ml of ampicillin and 30 μg/ml of chloramphenicol at 37 °C. Expression of the recombinant proteins was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), when the optical density of the culture at 600 nm reached 0.5–0.6. After 3 h of induction, the cells were harvested by centrifugation. The cell pellet (3 g of wet weight from 1 L culture) was resuspended in 25 ml of lysis buffer (20 mM HEPES, 150 mM KCl, pH 7.2) and lysed using a microfluidizer (M-110P; Microfluidics, USA). The insoluble materials of the lysate were then removed by centrifugation at 12,000*g* for 20 min, and the membrane fraction was precipitated by centrifugation at 100,000*g* for 1 h. The proteins in the membrane fraction were solubilized by incubation with 20 ml of lysis buffer containing 1% Sarkosyl for 1 h, and insoluble materials were removed by centrifugation at 100,000*g* for 1 h. The resulting supernatant was mixed with 2 ml of Ni-NTA for 1 h at 4 °C. After washing the resin with 10 ml of washing buffer (5 mM imidazole, 150 mM KCl, 20 mM HEPES, pH 7.2, 1% Sarkosyl), the proteins bound to the Ni-NTA resin were eluted using an elution buffer (200 mM imidazole, 150 mM KCl, 20 mM HEPES, pH 7.2, 1% Sarkosyl). The excess imidazole in the protein solution was removed using a desalting column.

Reconstitution of P9-5-HT_{3A} in liposomes

Large unilamellar liposomes (LUVs) with a diameter of 400 nm, consisting of POPC and POPS (weight ratio of POPC:POPS = 7:3) or POPC, POPS, and DOGS-NTA-Ni (weight ratio of POPC:POPS:-DOGS-NTA-Ni = 6.9:3:0.1) in buffer A (20 mM HEPES, 150 mM KCl, pH 7.2), were prepared using an extruder (Avanti, USA) as previously described [18]. Proteoliposomes were prepared by mixing 0.5 ml of LUVs (lipid concentration of 5 mg/ml) in buffer A with the same volume of P9-5-HT_{3A} in buffer A containing 1% Sarkosyl, at a 1:1,000 protein-to-lipid molar ratio. After incubation at 4 °C for 1 h, the detergent in the mixture was removed by treatment with 100 mg of Bio-Beads for 3 h at 4 °C twice, or by dialysis against a 1,000-fold volume of buffer A for 24 h with two dialysis buffer changes. The vesicles were recovered by ultracentrifugation at 110,000*g* for 20 min using an Airfuge (Beckman), and any attached proteins on the surface of proteoliposomes were removed by washing 7–8 times using ultra centrifugation. The proteoliposomes were then resuspended in 200 μl of buffer A. The amount of proteins in the recovered proteoliposomes was estimated

from the absorption at 280 nm, and with a BCA protein assay kit as described by the product manual (Pierce, IL, USA). The lipids in the proteoliposomes were assayed by measuring the amount of phosphates generated from acid hydrolysis, as previously described [19]. For the Ca^{2+}-release assay, the proteoliposomes were prepared by mixing 5 mg/ml of LUVs in buffer A containing 7.5 mM $CaCl_2$ with the same volume of P9-5-HT_{3A} in buffer A containing 1% Sarkosyl, at a 1:4,000 protein-to-lipid molar ratio, and the proteoliposomes were prepared after using Bio-Beads to remove the Sarkosyl, as described above.

Liposomal release assay

The channel activity of the reconstituted P9-5-HT_{3A} was examined by measuring the release of Ca^{2+} ions from the proteoliposomes using Fluo-3, a Ca^{2+} ion sensitive fluorescence dye. Fluo-3 (10 μM) and proteoliposomes (20 μg/ml) were mixed in buffer A (0.1 mL), and the release of Ca^{2+} ions was monitored using a Cary Eclipse Fluorescence Spectrophotometer (Varian, USA) at the excitation and emission wavelengths of 500 and 526 nm (slits, 5 nm), respectively, in a 1-cm path-length quartz cuvette at 25 °C. The extent of Ca^{2+} ion release was quantified on a percentage basis according to the following equation: $[(F_t - F_0) / (F_{100} - F_0) \times 100]$, where F_t is the measured fluorescence of reagent-treated LUVs at time t, F_0 is the average fluorescence of the LUV suspension for the initial 1–2 min before the addition of 5-HT, and F_{100} is the average fluorescence value of the final 1–2 min after the complete disruption of proteoliposomes by addition of Triton X-100 (final concentration, 0.1%).

Results

P9-5-HT_{3A} is highly expressed in the membrane fraction of E. coli as a fusion protein of the phi6 P9 protein

Phi6 is an enveloped bacteriophage that infects *Pseudomonas syringae*, and its P9 protein is a major envelope protein consisting of 90 amino acids with a single transmembrane helical region [20]. Previously, we have reported that human ET_A could be highly expressed in the membrane fraction in *E. coli* when ET_A was expressed as a P9 fusion protein [17]. To achieve this, the P9 protein was fused to the N-terminus of the wild-type or W178A mutant 5-HT_{3A} along with a His6 tag at the C-terminus, as shown in Fig. 1A. The W178A mutant was prepared in order to verify the serotonin-dependent ion-channel opening activity of P9-5-HT_{3A}, since the W178 residue, which is equivalent to W183 of mouse 5-HT_{3A}, is critical for serotonin-dependent ion-channel opening [21,22]. These fusion proteins were expressed in *E. coli* Rosetta2 (DE3) cells. High-level expression of the fusion protein in the membrane fraction after induction with 1 mM IPTG was observed in the Coomassie blue-stained gel following SDS–PAGE (Fig. 1B, lanes 1 and 2). The induced protein was confirmed as P9-5-HT_{3A} by western blot analysis using anti-His6 antibody (Fig. 1B, lanes 3 and 4). The average expression level of P9-5-HT_{3A} was 1.5–2 mg per liter of culture, indicating that the P9 fusion system was very effective for the high-level expression of 5-HT_{3A} in the membrane fraction of *E. coli*.

Expressed P9-5-HT_{3A} was purified using a simple affinity column

To purify the expressed P9-5-HT_{3A} in the membrane fraction, the efficiency of various detergents for the solubilization of P9-5-HT_{3A} was examined. The membrane fraction from

the lysate of cells expressing P9-5-HT_{3A} was treated with *n*-dodecyl β-d-malto-side (D), lauryldimethylamine oxide (L), *n*-octyl-β-d-glucopyranoside (O), Triton X-100 (X), or Sarkosyl (S). After ultracentrifugation at 100,000*g* for 1 h, the supernatant and pellet fractions were analyzed by western blotting using anti-His tag antibody. As shown in Fig. 1C, the P9-5-HT_{3A} in the membrane fraction was completely solubilized in 1% Sarkosyl, and about 50% was solubilized in 1% lauryldimethylamine oxide. However, P9-5-HT_{3A} was poorly solubilized in the other detergents. The solubilized P9-5-HT_{3A} was further purified, using a simple Ni-NTA affinity column, to a purity of >90% (Fig. 1D, lane 4). The purified P9-5-HT_{3A} ran as a 56-kDa protein in SDS–PAGE, which was slightly lower than the calculated size (67 kDa). Membrane proteins such as GPCRs often migrate in SDS–PAGE faster than their actual size [17]. The identity of the purified protein as P9-5-HT_{3A} was further confirmed by western blotting using anti-His6 antibody (data not shown). Finally, about 1.0 mg of purified protein was obtained from a 1 L culture. These results indicate that P9-5-HT_{3A} was highly expressed in the membrane fraction of *E. coli* and was effectively purified by Ni-NTA affinity chromatography.

Purified P9-5-HT_{3A} presents as oligomers in detergent micelles

It was well established that ligand-gated ion channels, such as nAChR, form pentamers. To investigate the oligomeric state of the purified P9-5-HT_{3A}, the size of the Ni-NTA affinity-purified protein was analyzed by gel filtration. At least four protein peaks were observed in the chromatogram (Fig. 2A). When the proteins from these peaks were analyzed by SDS–PAGE, only the P9-5-HT_{3A} band was observed in these peaks (Fig. 2A, upper panel), indicating that these peaks in the gel filtration chromatogram represented different oligomeric states of P9-5-HT_{3A}. The sizes of these peaks were estimated to 78, 125, 223, and 366 kDa, respectively, based on the relative elution volumes of marker proteins (Fig. 2B). It was suggested that P9-5-HT_{3A} tightly retains the micelles of Sarkosyl, which resulted in an increase of apparent molecular weight. Considering the predicted size of monomeric P9-5-HT_{3A} (67.2 kDa) and the micelles of Sarcosyl (0.6 kDa), these peaks corresponded to a monomer, dimer, trimer, and pentamer, respectively. These results indicated that the purified proteins presented as a mixture of oligomers, including a pentamer.

Reconstituted P9-5-HT_{3A} in proteoliposomes has the ligand-dependent ion-channel activity

To prepare the proteoliposomes containing the purified P9-5-HT_{3A}, we used Bio-Beads adsorption and dialysis to remove the detergent. Additionally, the effect of NTA-Ni-containing lipids (DOGS-NTA-Ni) on the incorporation of the His-tag-containing P9-5-HT_{3A} into proteoliposomes was examined. Between the two methods, the Bio-Beads adsorption method clearly showed a much higher recovery rate of P9-5-HT_{3A} and lipids than the dialysis method. About 9% of proteins and 50% of lipids were recovered in the proteoliposomes prepared by the Bio-Beads method, whereas only 1.6% and 2.3% of proteins and lipids, respectively, were recovered from the dialysis method (Table 1). Furthermore, when a small portion of NTA-Ni-linked lipid (DOGS-NTA-Ni) was incorporated in the liposomes, the recovery rate of 5-HT_{3A} in the proteoliposomes increased to 12.3%, suggesting that the specific interaction between the His6 tag of P9-5-HT_{3A} and the NTA-Ni group in the liposomes enhanced the reconstitution of P9-5-HT_{3A}.

The expressed 5-HT_{3A} gene in oocytes or mammalian cells showed characteristics similar to those of the native 5-HT_3 receptor channel [2,23], and the permeability of divalent cations such as Ca^{2+} through the expressed 5-HT_3 ion channel was comparable to that of monovalent cations such as Na^+ or K^+ [24], suggesting that a properly constituted 5-HT_{3A} could transport Ca^{2+} ions. To examine whether or not the reconstituted P9-5-HT_{3A} has ion-transport activity, the release of Ca^{2+} ions through P9-5-HT_{3A} in Ca^{2+}-encapsulated proteoliposomes was measured using Fluo-3 (Fig. 3A), a Ca^{2+} ion-sensitive fluorescent dye [25]. Addition of 10 μM or higher concentration of serotonin to the proteoliposome solution evoked prompt release of the Ca^{2+} ion, the amount of which corresponded to 7% of the total amount of encapsulated Ca^{2+} (Fig. 3B). In contrast, the P9-5-HT_{3A}(W178A) mutant showed no significant Ca^{2+} release after treatment of up to 20 μM of 5-HT (Fig. 3C). These results indicate that the reconstituted P9-5-HT_{3A} in proteoliposomes had serotonin-dependent ion-channel activity, and the effect of a mutation that defects this serotonin-dependent ion-channel activity of 5-HT_{3A} [21,22] was easily observed by assaying the Ca^{2+} ions released from the proteoliposomes. The effective concentration of serotonin for opening the P9-5-HT_{3A} ion channel was within 5–10 μM (Fig. S1), which was similar to the reported EC_{50} values for 5-HT (2–4 μM) [26,27]. The fact that the percentage of Ca^{2+} release relative to the total amount of Ca^{2+} released by detergent treatment was about 7% perhaps indicates that only 7% of the prepared proteoliposomes contained functional P9-5-HT_{3A}. To further confirm that the reconstituted P9-5-HT_{3A} has serotonin-dependent ion-channel activity, the inhibitory effect of ondansetron, an antagonist of 5-HT_{3A} [28,29], on the release of Ca^{2+} from the proteoliposomes by 5-HT was examined. The ion-channel activity of P9-5-HT_{3A} was not affected by the treatment of 1.0 μM of ondansetron (Fig. S2). When the proteoliposomes were incubated with 10–1000 nM of ondansetron, however, the release of Ca^{2+} from the proteoliposomes by 20 μM 5-HT was significantly reduced (Fig. 3D). These results indicate that P9-5-HT_{3A} was functionally reconstituted in proteoliposomes and could bind specifically to 5-HT as well as to ondansetron.

Discussion

High-level expression of multi-transmembrane helical proteins such as channels or GPCRs has been scarcely reported. Some membrane proteins expressed as fusion proteins containing a well-folded protein such as GFP or thioredoxin have shown increased expression [15,16]. However, such an expression system might be applicable only in specific cases. In this report, we used a small single transmembrane helical protein, P9, as a fusion partner for the expression of human 5-HT_{3A}. P9-5-HT_{3A} showed high-level expression in *E. coli*, and more than 50% of the expressed protein was located in the membrane fraction. The P9 protein is a major envelope protein of bacteriophage phi6, and it was highly expressed in the membrane fraction of *E. coli* when using the T7 promoter (data not shown). The initial high rate of expression and insertion into the membrane might assist the high-level expression of multi-transmembrane proteins fused to the P9 protein.

The purified P9-5-HT_{3A} appeared as a mix of oligomers (Fig. 2A) rather than a unique population of the pentamer, which was observed in 5-HT_{3A} expressed in mammalian cells [5,30]. The ligand-dependent ion-channel activity of P9-HT_{3A}, however, was observed when it was reconstituted into liposomes. An electrophysiological analysis indicated that Na^+, K^+,

and Ca^{2+} ions are permeable through the 5-HT_3 ion channel, which has a pore size of 7.6 Å [24]. When the oligomers of purified P9-5-HT_{3A} were reconstituted into proteoliposomes, only 7% of the proteoliposomes showed serotonin-dependent Ca^{2+} ion release (Fig. 3B), suggesting that only a minor fraction of P9-5-HT_{3A}, probably the pentameric form, might form functional ion channels. It had been shown that glycosylation was important for the proper folding of 5-HT_3. Deglycosylation of active 5-HT_3 expressed in insect cells did not reduce its ability to bind ligands but did assist in the proper folding of 5-HT_3 [31]. Hence, a minor fraction of P9-5-HT_{3A} subunits expressed in *E. coli* would form an active pentamer. Optimization of the folding condition, or application of an *E. coli* strain that can afford glycosylation, would enhance the yield of properly folded P9-5-HT_{3A}.

The serotonin-dependent release of Ca^{2+} ions from the proteoliposomes reconstituted with P9-5-HT_{3A} and the inhibition of the P9-5-HT_{3A} ion channel by ondansetron indicated that the Ca^{2+} release assay could be applied to examine the activity of P9-5-HT_{3A}. Calcium ions have shown an inhibitory effect on the 5-HT_3 ion channel [32]. However, the calcium ion binding site is located in the extracellular region of 5-HT_3 [11], and the calcium ions presenting only inside of the proteoliposomes could not affect the calcium-sensing site of the reconstituted P9-5-HT_{3A}, which was located outside of the proteoliposomes. This Ca^{2+} release assay with the reconstituted P9-5-HT_{3A} could be used as a model system for the structural and functional analyses of the 5-HT_3 ion channel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pep.2013.01.001.

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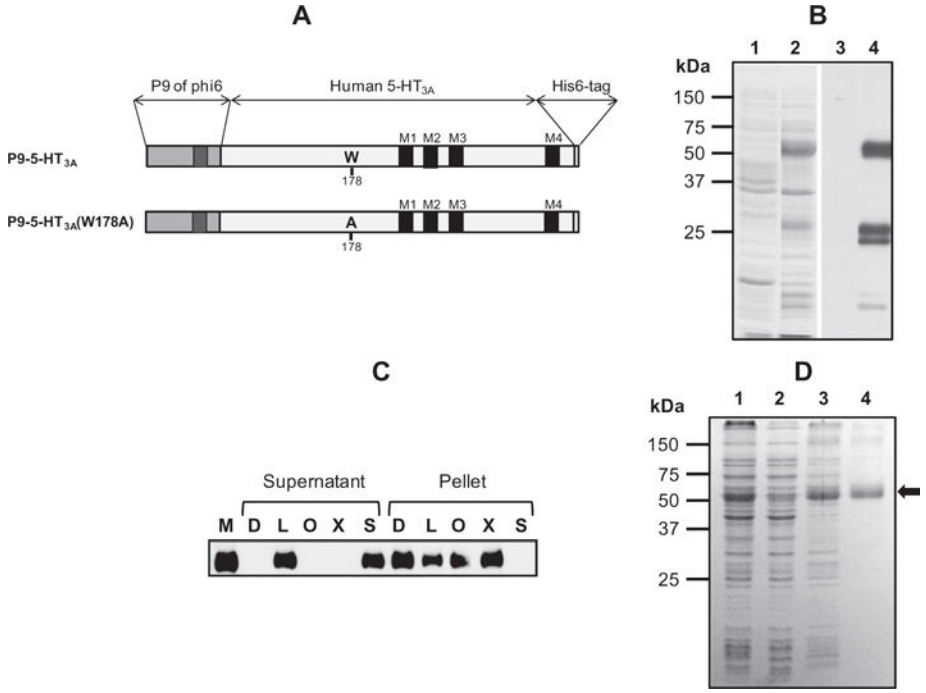

Fig. 1.

Expression and purification of P9-5-HT_{3A}. (A) Schematic representation of wild-type and W178A mutant 5-HT_{3A} along with the P9 and His6 tag flanking the N- and C-termini of 5-HT_{3A}. The transmembrane regions in P9 and 5-HT_{3A} are indicated as black rectangles, and the position of W178 or A178 is indicated. (B) The expression level of P9-5-HT_{3A} in *E. coli* was examined by SDS–PAGE and western blotting. The membrane fractions of *E. coli* before (lanes 1 and 3) and after (lanes 2 and 4) induction with 1 mM IPTG were analyzed by SDS–PAGE, and the gel was stained with Coomassie blue dye (lanes 1 and 2) or subjected to western blotting using anti-His tag antibody (lanes 3 and 4). (C) The solubility of expressed P9-5-HT_{3A} in various detergents was examined. The membrane fraction (M) from the lysate of cells expressing P9-5-HT_{3A} was treated with 1% *n*-dodecyl β-D-maltoside (D), 1% lauryldimethylamine oxide (L), 2% *n*-octyl-β-d-glucopyranoside (O), 1% Triton X-100 (X), or 1% Sarkosyl (S) for 1 h at 4 °C. After ultracentrifugation at 100,000*g* for 1 h, the proteins in the supernatant and pellet fractions were separated by SDS–PAGE and analyzed by western blotting using anti-His tag antibody. D. SDS–PAGE analysis during the purification of P9-5-HT_{3A}. Lane 1, cell lysate; lane 2, soluble fraction from cell lysate after ultracentrifugation; lane 3, membrane fraction; lane 4, purified protein after Ni-NTA column chromatography. Purified P9-5-HT_{3A} is indicated as an arrow.

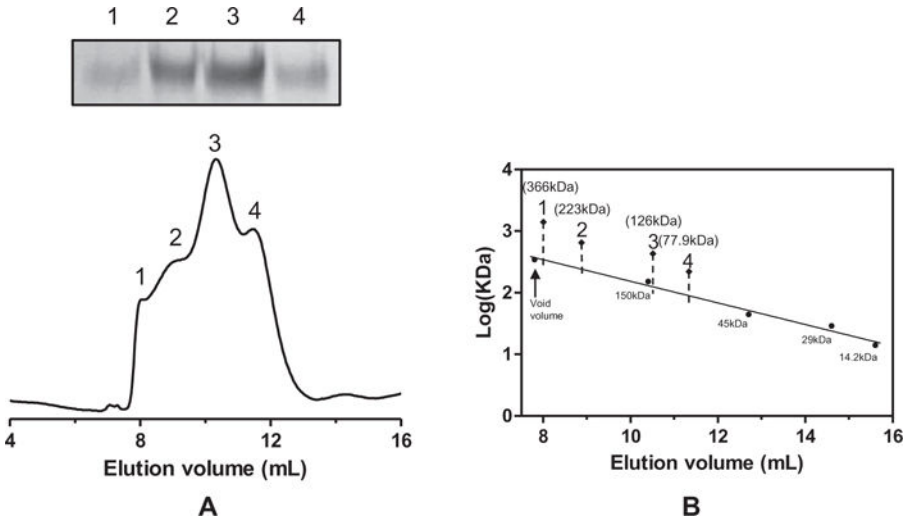

Fig. 2.

Gel filtration chromatography of P9-5-HT_{3A}. (A) Purified P9-5-HT_{3A} was injected onto a Superdex 200HR 10/300 GL gel filtration column equilibrated in 0.5% Sarkosyl, 50 mM HEPES, and 150 mM KCl, pH 7.2. The column was run at 0.5 ml/min, and the protein elution profile was monitored at 280 nm. The indicated fractions (1–4) were also analyzed by SDS–PAGE (upper panel). (B) The size of P9-5-HT_{3A} was estimated using marker proteins (14.2, 29, 45, and 150 kDa).

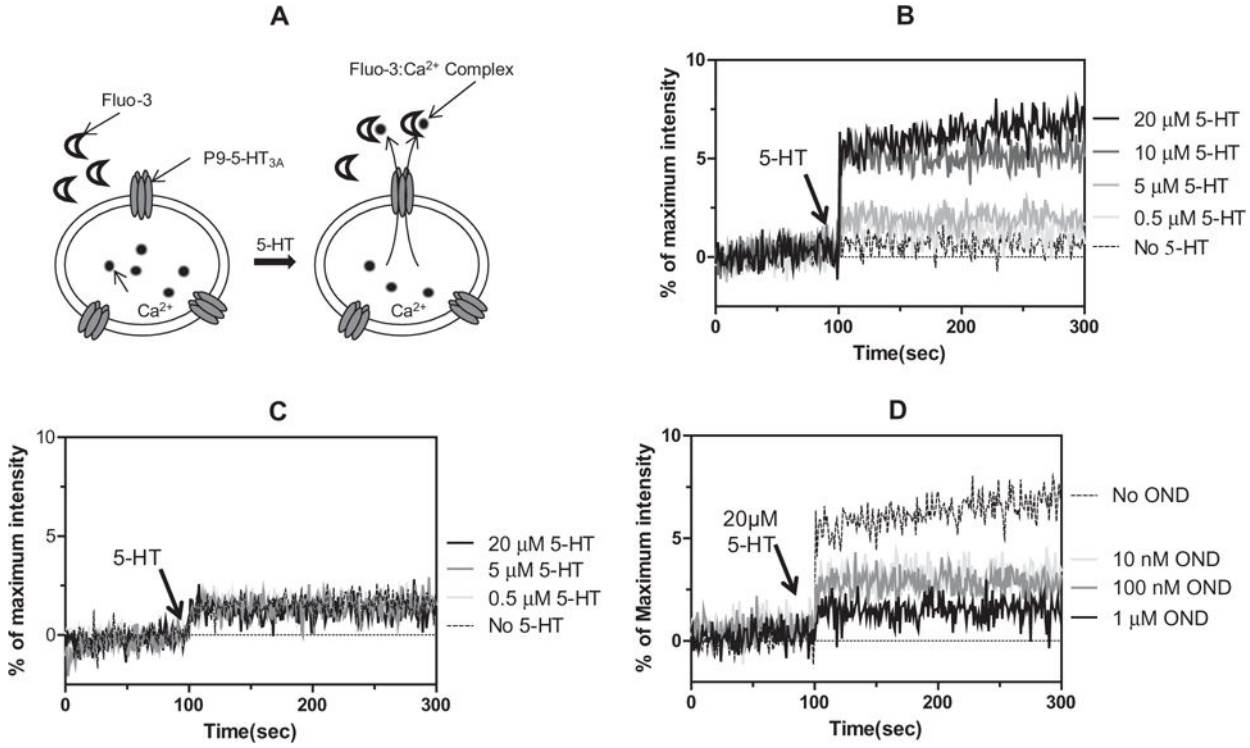

Fig. 3.

Calcium release assay of proteoliposomes containing P9-5-HT_{3A}. (A) Schematic representation of the serotonin-dependent ion-channel opening assay with proteoliposomes reconstituted with P9-5-HT_{3A}. The Ca^{2+} ions released from the P9-5-HT_{3A} ion channel bind to Fluo-3, and the enhanced fluorescence of the Fluo-3:Ca^{2+} complex was measured. (B) The percentage release of Ca^{2+} from proteoliposomes containing wild-type P9-5-HT_{3A} was measured by mixing different concentrations of serotonin (5-HT) at 25 °C. The time point of the addition of 0 M (dotted line), 0.5 μM (light grey line), 5 μM (grey line), 10 μM (dark grey line), or 20 μM (black line) 5-HT is indicated by the arrow. (C) Percentage release of Ca^{2+} from proteoliposomes containing P9-5-HT_{3A}(W178A). The time point of the addition of 0 M (dotted line), 0.5 μM (light grey line), 5 μM (grey line), or 20 μM (black line) 5-HT is indicated by the arrow. (D) The percentage release of Ca^{2+} from proteoliposomes that were preincubated with 0 μM (dotted line), 10 nM (light grey line), 100 nM (grey line), or 1 μM (black line) ondansetron was measured at 25 °C. The time point of the addition of 20 μM 5-HT is indicated by the arrow.

Table 1

Reconstitution efficiency of P9-5-HT_{3A} in proteoliposomes.

Detergent removal methods	Lipid composition[a] (POPC:POPS:DOGS-NTA-Ni)	Recovery (%)[b]	
		Protein	Lipids
Bio-Beads	7:3:0	9.3	49.8
	6.9:3:0.1	12.3	53.4
Dialyis	7:3:0	1.6	2.3

[a]The mass ratio of POPC, POPS, and DOGS-NTA-Ni.

[b]The percentage of recovered P9-5-HT_{3A} and lipids in the proteoliposomes compared with the initial amounts of P9-5-HT_{3A} and lipids.