Promiscuous coassembly of serotonin 5-HT₃ and nicotinic α4 receptor subunits into Ca²⁺-permeable ion channels

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ABSTRACT Serotonin (5-hydroxytryptamine) type 3 receptors (5-HT₃R) and nicotinic acetylcholine receptors are structurally and functionally related proteins, yet distinct members of the family of ligand-gated ion channels. For serotonin (5-H₃R) and nicotinic acetylcholine receptor α4 subunits coassemble into a novel type of heteromeric ligand-gated ion channel, which is activated by 5-HT. The Ca²⁺ permeability of this heteromeric ion channel is enhanced as compared with that of the homomeric 5-HT₃R channel. Heteromeric 5-HT₃R/α4 homomeric 5-HT₃Rs have similar pharmacological profiles, but distinct sensitivities to block by the antagonist 7-tubocurarine. Coassembly of subunits beyond the boundaries of ligand-gated ion channel families may constitute an important mechanism contributing to the diverse properties and functions of native neurotransmitter receptors.

Through molecular cloning a plethora of subunits of ligand-gated ion channels has been identified. In heterologous expression systems, the coassembly of related subunits permits formation of a vast repertoire of neurotransmitter receptors, each with its own characteristic properties. A focus in contemporary neuroscience is to understand the functional significance of the variety of cloned subunits for the diverse properties and functions of native neurotransmitter receptors. For serotonin (5-hydroxytryptamine) type 3 receptors (5-HT₃R), little molecular diversity has become apparent: only a single class of 5-HT₃ subunit has been cloned (1–3), and these subunits form homomeric receptors with similar functional properties in heterologous expression systems (4, 5). Conversely, studies of native receptors, indicating substantial heterogeneity of 5-HT₃R properties, suggest the existence of additional subunits involved in the formation of heteromeric 5-HT₃Rs (6–9).

Ligand-gated ion channels share homology in their primary and predicted secondary structures. A particularly close relationship between 5-HT₃R and nicotinic acetylcholine receptors (nAChR) has been demonstrated by the construction of a functional chimeric receptor, containing the ligand-binding domain of the nAChR α7 and the ion channel domain of the 5-HT₃R subunits (10). Because of the close resemblance between 5-HT₃ and nAChR subunits, we examined whether the 5-HT₃R subunit can coassemble with nAChR subunits to form heteromeric receptors.

MATERIALS AND METHODS

Expression and Recording from Xenopus Oocytes. Oocytes from mature specimens of Xenopus laevis were harvested, injected, and incubated as described before (11). cDNA encoding the 5-HT₃R-A and cDNAs encoding the α2, α3, α4, α7, β2, and β4 nAChR subunits were injected into the nucleus either alone or pairwise at a ratio of 1:3 5-HT₃R-A/nAChR cDNA (total injection volume ~32 nl). For experiments under Cl⁻-free conditions, oocytes were incubated in Cl⁻-free modified Barth’s solution as described before (11). Ion currents were recorded from oocytes 2–5 days after injection by conventional two-microelectrode voltage clamp. The membrane potential was held at −60 mV or at −20 mV, unless otherwise noted. Microelectrodes (≥1 MΩ) were filled with 3 M KCl, or with 3 M K-methanesulfonate and 50 mM KCl for experiments performed under Cl⁻-free conditions. Oocytes were continuously superfused with external solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 10 mM Hepes, pH 7.2 with NaOH. For recordings under Cl⁻-free conditions the same external solution was used with methanesulfonate substituting for Cl⁻. For recordings under Ca²⁺-free conditions external solution with 1 mM Mg²⁺ substituting for Ca²⁺ was used. For chelation of intracellular Ca²⁺, 50 nl of a 50 mM BAPTA [bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid] solution was injected into oocytes during the experiments via a third micropipette by using a Drummond microinjector. All agonists used were applied at near maximum-effective concentrations (5, 12).

Expression and Immunoprecipitation from Human Embryonic Kidney (HEK) 293 Cells. HEK 293 cells were transfected by calcium phosphate precipitation (13) by using the eukaryotic expression vector pRC/CMV containing cDNA for the 5-HT₃R, the nAChR α4, the nAChR β2, or the myc-tagged γ-aminobutyric acid type A receptor (GABA₄) α1 subunit. After transfection cells were incubated for 72 hr at 3% CO₂. Preparation of membranes from transiently transfected cells, solubilization of membranes, and concentration of membranes for direct immunoblots were performed according to previously described methods (14). Receptors were immunoprecipitated by using 5 µg of subunit-specific antibody, followed by collection using a mixture of protein A and G-Sepharose.

This paper was submitted directly (Track II) to the Proceedings office. Abbreviations: 5-HT, serotonin (5-hydroxytryptamine); 5-HT₃R, serotonin type 3 receptor; BAPTA, bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; GABA₄, γ-aminobutyric acid type A receptor; mCPBG, meta-chlorophenylbiguanide; ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; HEK, human embryonic kidney.

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RESULTS

5-HT₃R and nAChR subunits were coexpressed in *Xenopus* oocytes and the agonist-evoked ion currents were compared with those in oocytes expressing homomeric 5-HT₃Rs. Inward currents induced in oocytes expressing homomeric 5-HT₃Rs by superfusion with 10 μM 5-HT were similar to those described previously (1, 4, 5, 16) (Fig. 1A). Application of 5-HT to oocytes, coinfected with 5-HT₃R subunit cDNA and cDNA encoding nAChR subunits α₂, α₃, α₇, β₂, or β₄, yielded responses indistinguishable from those of homomeric 5-HT₃Rs. However, the combination of 5-HT₃R and α₄ subunits yielded a biphasic response at a holding potential of −20 mV (24 of 24 oocytes, eight frogs), whereas the ion current mediated by homomeric 5-HT₃Rs was always monophasic (Fig. 1A). Oocytes injected with α₄ cDNA alone did not respond to 5-HT nor to acetylcholine (ACh), although the amount of α₄ cDNA was sufficient for the expression of functional heteromeric nAChR when coinfected with β₂ cDNA (Fig. 1B). These results show that coexpression of nAChR α₄ subunits with 5-HT₃R subunits alters the properties of the 5-HT₃R-mediated ion current and suggest that this is caused by coassembly of 5-HT₃R and α₄ subunits.

Formation of heteromeric 5-HT₃/α₄ receptors was demonstrated by transiently transfecting plasmids encoding these two subunits into *HEK 293* cells followed by immunoprecipitation by using a mAb to the α₄ subunit (mAb 299). Under the conditions used, mAb 299 will immunoprecipitate complete receptors, whose subunit size and composition then can be revealed by further analysis. By using denaturing gels followed by Western blots, labeling of the mAb 299-precipitated product by both mAb 299 and a polyclonal 5-HT₃R-specific antibody is demonstrated (Fig. 2A). This result shows that the original protein contained both 5-HT₃R and α₄ subunits. Immunoprecipitation applied to cells that were transfected with plasmids containing either the α₄ subunit or the 5-HT₃R subunit did not result in protein labeled by the alternative antibody (Fig. 2A). Control experiments, using the nAChR β₂ subunit-specific mAb 270 (17) under identical conditions as above, show that nAChR α₄ and β₂ subunits coprecipitate. This finding is consistent with the notion that α₄ and β₂ subunits form functional heteromeric nAChR. Conversely, neither the 5-HT₃R subunit nor the nAChR α₄ subunit coprecipitate with the myc-tagged GABAₐ₃R α₁ subunit (Fig. 2B). These results demonstrate the specific coassembly of 5-HT₃R and nAChR α₄ subunits into heteromeric receptors.

The biphasic nature of 5-HT₃/α₄ receptor-mediated ion current in oocytes depends on the presence of Ca²⁺ and Cl⁻. Currents evoked in oocytes expressing 5-HT₃/α₄ receptors in the absence of either Ca²⁺ or Cl⁻ are monophasic and indistinguishable from the currents mediated by homomeric 5-HT₃Rs (6 of 6 oocytes, three frogs; Fig. 3). This finding indicates that 5-HT₃/α₄ channel opening results in Ca²⁺ entry and secondary activation of Ca²⁺-dependent Cl⁻ channels, which are natively expressed in *Xenopus* oocytes (18). Attempts to confirm Ca²⁺ entry upon activation of 5-HT₃/α₄ receptors in oocytes by using Fura-2 Ca²⁺ imaging did not result in detectable signals, whereas injection of inositol 1,4,5-
trisphosphate into the same oocytes gave robust increases in intracellular \([\text{Ca}^{2+}]\) (not shown). This finding suggests that \(\text{Ca}^{2+}\) entry is local and that amounts of \(\text{Ca}^{2+}\) were too low to be detected, consistent with the earlier observation that activation of homomeric 5-HT3/\(\alpha_4\) receptors causes only modest Fura-2 signals in oocytes (16). However, the \(\text{Ca}^{2+}\) entry through heteromeric 5-HT3/\(\alpha_4\) receptors was confirmed by using the \(\text{Ca}^{2+}\) chelator BAPTA. The \(\text{Ca}^{2+}\)-dependent component of the biphasic current was completely abolished after intracellular injection of BAPTA (3 of 3 oocytes, two frogs; Fig. 4A). Moreover, enhanced \(\text{Ca}^{2+}\) entry through heteromeric 5-HT3/\(\alpha_4\) receptors was directly demonstrated by using Fura-2 \(\text{Ca}^{2+}\) imaging in HEK 293 cells expressing homomeric 5-HT3/\(\alpha_4\) receptors. The maximum receptor-mediated \(\text{Ca}^{2+}\) entry in HEK 293 cells expressing 5-HT3/\(\alpha_4\) receptors was \(\sim 2\)-fold enhanced from 329 ± 18 nM to 594 ± 18 nM, respectively (Fig. 4B; \(n = 6\)). The values of the EC50 and Hill coefficients obtained from the concentration-effect curves of mCPBG on homomeric and heteromeric receptors were indistinguishable (Fig. 4B; 111 ± 16 nM and 3.3 ± 1 for homomeric receptors; 97 ± 5 nM and 4.1 ± 0.9 for heteromeric receptors, \(n = 6\)). The combined electrophysiological and \(\text{Ca}^{2+}\) imaging data demonstrate that the \(\text{Ca}^{2+}\) permeability of heteromeric 5-HT3/\(\alpha_4\) receptors is significantly enhanced as compared with that of homomeric 5-HT3Rs.

The pharmacological profiles of the 5-HT3 and 5-HT3/\(\alpha_4\) receptors are very similar. The concentration-effect curves of 5-HT are indistinguishable (Fig. 5A), with EC50 and Hill coefficients of 2.8 ± 0.2 \(\mu\)M and 2.8 ± 0.3 for homomeric receptors, and 2.8 ± 0.5 \(\mu\)M and 2.7 ± 0.3 for heteromeric receptors (\(n = 3\)). Homomeric and heteromeric receptors are activated by the 5-HT3R agonists 2-methyl-5-HT, dopamine, and mCPBG (Fig. 5B), and not by the nAChR agonists ACh...
Fig. 3. Enhanced Ca\(^{2+}\) permeability of heteromeric 5-HT\(_3/a4\) receptors expressed in oocytes. Ion currents mediated by homomeric 5-HT\(_3\)Rs (Left) and heteromeric 5-HT\(_3/a4\) receptors (Right) were evoked at holding potentials of −60, −20, and +20 mV (bottom to top traces) with 10 \(\mu\)M 5-HT under control, Ca\(^{2+}\)-free, and Cl\(^{-}\)-free conditions (see Materials and Methods). Traces recorded under Ca\(^{2+}\)-free conditions were obtained from the same oocytes used to record control traces. (Insets) Magnified current traces recorded at the holding potential of −20 mV.

Tetramethylammonium, nicotine, and physostigmine (not shown). The nAChR agonist cytisine acts as a partial agonist on 5-HT\(_3\) and 5-HT\(_3/a4\) receptors, with an efficacy of 3–5\% (Fig. 5B). None of the agonists discriminates between homomeric and heteromeric receptors. Both receptors also are inhibited by a low concentration of the selective 5-HT\(_3\)R antagonist MDL 72222 (Fig. 5B). The 5-HT\(_3\)R and nAChR antagonist d-tubocurarine is less potent in blocking heteromeric than homomeric receptors (Fig. 5B and C). The IC\(_{50}\) of d-tubocurarine on the 5-HT\(_3/a4\) receptor (2.4 ± 0.4 nM) is significantly higher than that on the 5-HT\(_3\)R (0.8 ± 0.2 nM; \(n = 3\); Student’s \(t\) test: \(P = 0.02\)). The Hill coefficients are indistinguishable (−1.1 ± 0.1 and −0.9 ± 0.1, respectively).

**DISCUSSION**

The results demonstrate that 5-HT\(_3\)R and nAChR \(\alpha4\) subunits coassemble into a novel type of heteromeric 5-HT\(_3\) receptor channel with enhanced Ca\(^{2+}\) permeability and reduced sensitivity to the antagonist d-tubocurarine as compared with the homomeric 5-HT\(_3\)R receptor-gated ion channel. These findings have significant implications, both for 5-HT\(_3\)R pharmacology and function and for ligand-gated ion channels in general. Since the cloning of the 5-HT\(_3\)R subunit in 1991 (1), no additional class of 5-HT\(_3\)R subunit has been found. There has been some interest in long and short splice variants of the 5-HT\(_3\)R subunit that have been identified in rodent, but not human, tissues (2, 3, 19, 20). In heterologous expression systems both subunits form functional, homomeric ligand-gated ion channels with similar pharmacological and biophysical properties (4, 5). Despite the limited molecular diversity of the 5-HT\(_3\)R, several lines of evidence suggest that at least some native 5-HT\(_3\)Rs are not solely composed of either long or short 5-HT\(_3\)R subunits. For example, the pharmacological profiles of 5-HT\(_3\)Rs composed of either long or short subunits are different from those of 5-HT\(_3\)Rs native to N1E-115 neuroblastoma cells (5, 9). In addition, 5-HT\(_3\)Rs in membranes from mouse brain and ileum appear to have different pharmacological profiles (7). The present finding, that it is possible to form heteromeric 5-HT\(_3\)R with properties distinct from those of homomeric 5-HT\(_3\)Rs, provides a lead for further investigations into the subunit composition and heterogeneity of native 5-HT\(_3\)Rs. As shown in Fig. 1, coexpression of nAChR \(\alpha2\), \(\alpha3\), \(\alpha7\), \(\beta2\), and \(\beta4\) subunits with the 5-HT\(_3\)R subunit did not alter specific kinetic properties of the 5-HT-induced ion current. However, the possibility that these nAChR subunits also coassemble with the 5-HT\(_3\)R subunit cannot be excluded at present.

Coassembly of 5-HT\(_3\)R with \(\alpha4\) subunits results in ion channels with enhanced Ca\(^{2+}\) permeability as compared with homomeric 5-HT\(_3\)Rs. Because of the importance of Ca\(^{2+}\) in cellular signaling, there has been considerable discussion in the literature about the level of Ca\(^{2+}\) entry through 5-HT\(_3\)Rs. It has previously been reported that the Ca\(^{2+}\) permeability of 5-HT\(_3\)Rs in N18 cells is relatively high (21). However, in a more recent study of recombinant 5-HT\(_3\)Rs expressed in oocytes no increase in Fura-2 fluorescence was found (16). Conversely, recombinant 5-HT\(_3\)Rs expressed in HEK 293 cells, and 5-HT\(_3\)Rs native to N1E-115 cells, do mediate an increase in intracellular Ca\(^{2+}\) detectable by Fura-2 (15). As the present results confirm that Ca\(^{2+}\) signals induced by 5-HT\(_3\)R activation in oocytes are much weaker than in HEK cells, the inconsis-

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**FIG. 4.** Heteromeric 5-HT\(_3/a4\) receptors mediate an increase in intracellular \([\text{Ca}^{2+}]_i\). (A) Ion currents evoked with 10 \(\mu\)M 5-HT (filled bar) at −20 mV, before and 5 min after intracellular injection of the Ca\(^{2+}\) chelator BAPTA. The superimposed traces were obtained from the same oocyte. (B) Fura-2 fluorescence concentration-effect curves of mCPBG-induced increases in \([\text{Ca}^{2+}]_i\), in HEK 293 cells expressing homomeric 5-HT\(_3\)Rs (■) or heteromeric 5-HT\(_3/a4\) receptors (○). Data points are mean ± SD of six cells. Absence of error bars indicates that the SD is smaller than the symbol size. (Insets) The increase in \([\text{Ca}^{2+}]_i\) evoked with 1 \(\mu\)M mCPBG in HEK 293 cells expressing homomeric 5-HT\(_3\)Rs (Lower) or heteromeric 5-HT\(_3/a4\) receptors (Upper).
In conclusion, this study demonstrates coassembly of authentic subunits belonging to distinct classes of neurotransmitter receptors. Previous studies have shown that within receptor families constraints on subunit interactions limit the kind of receptor species produced (32, 33). The constraints applying to coassembly of subunits from different families remain to be determined. However, promiscuous coassembly beyond the boundaries of ligand-gated ion channel families creates another level of diversity for this family of proteins.

Note added in proof: Recently, a paper has appeared in which the authors report that they could not detect coprecipitation of 5-HT3R and nAChR subunits from pig cerebral cortex homogenates (34). Whether promiscuous coassembly of 5-HT3R and nAChR subunits occurs in other brain areas or in small fractions of specific cell populations remains to be determined.

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