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Short communication

Comparison of [^{3}H]YM060 binding to native and cloned rat 5-HT$_3$ receptors

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Abstract

We characterized [^{3}H]YM060 ([*methyl*-^{3}H]-(-)-(*R*)-5-[(methyl-1*H*-indol-3-yl)carbonyl]-4,5,6,7-tetrahydro-1*H*-benzimidazole monohydrochloride) binding in membrane homogenates prepared from three different rat tissues (cerebral cortex, ileum and colon), and compared the binding characteristics between the native and cloned rat 5-HT$_3$ receptors. The dissociation constant (K_d) of [^{3}H]YM060 was similar in all membranes. In competition studies, the affinity of 5-HT$_3$ receptor agonists and antagonists was similar between the native and the cloned rat 5-HT$_3$ receptors. In conclusion, intra-species difference of 5-HT$_3$ receptor was not observed in rats and pharmacological properties of the cloned rat 5-HT$_3$ receptor were nearly identical to that of the native rat 5-HT$_3$ receptor.

Keywords: 5-HT$_3$ receptor; [^{3}H]YM060 ([*methyl*-^{3}H]-(−)-(*R*)-5-[(1-methyl-1*H*-indol-3-yl)carbonyl]-4,5,6,7-tetrahydro-1*H*-benzimidazole monohydrochloride); Cerebral cortex; Ileum; Colon; 5-HT$_3$ receptor, cloned, rat

1. Introduction

The 5-HT$_3$ receptor is a member of the ligand-gated ion channel family, which includes nicotinic acetylcholine, GABA$_A$ and glycine receptors (Boess and Martin, 1994). Although other members of the ligand-gated ion channel family consist of many different subunits forming a heteromeric complex (Ortells and Lunt, 1995), only a single subunit of the 5-HT$_3$ receptors has been cloned from mouse cell line NCB-20 (Maricq et al., 1991), rats (Isenberg et al., 1993) and humans (Miyake et al., 1995). Johnson et al. (1995) reported that mRNA encoding the A subunit of the 5-HT$_3$ receptor in rat brain was also expressed in the small intestine of rats.

While an inter-species difference in 5-HT$_3$ receptors has been confirmed by ligand binding affinities and electrophysiological characteristics (Hoyer et al., 1994), little is known about the existence of an intra-species difference in 5-HT$_3$ receptors. Recently, Bonhaus et al. (1993) reported that the affinity of RS-42358-197, YM060 and *m*-chlorophenylbiguanide is different between brain and ileal membranes in mice when using [^{3}H]RS-42358-197. Perren et al. (1995), however, found no clear evidence of an intra-species difference in mouse tissues using [^{3}H]granisetron.

[^{3}H]YM060 is a potent and selective 5-HT$_3$ receptor radioligand (Akuzawa et al., 1995). In the present study, [^{3}H]YM060 binding properties were examined in membrane homogenates prepared from three different tissues (cerebral cortex, ileum and colon) within a single strain to clarify the intra-species difference in 5-HT$_3$ receptors. Furthermore, [^{3}H]YM060 binding in the native tissues was compared with that in cloned rat 5-HT$_3$ receptors expressed in COS-1 cells.

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2. Materials and methods

2.1. Tissue preparation

Male Wistar rats weighing 250–300 g were used. Tissue preparation was performed by the method of Kilpatrick et al. (1991). The rats were decapitated, their brains removed and the cerebral cortex dissected. Their ileum and colon were also dissected. The tissues were finely minced with scissors and homogenized in 30 volumes of ice-cold 50 mM Hepes buffer (pH 7.4 at 4°C) with a Polytron (Kinematica, Lucerne, Switzerland). The homogenate was centrifuged at $48\,000 \times g$ for 30 min. The pellet was resuspended in Hepes buffer and recentrifuged as above. The final pellet from brain tissue was suspended in 30 volumes of Hepes buffer and from ileum or colon tissue in 10 volumes of Hepes buffer. The ileum and the colon tissue were filtered through nylon mesh before using in the binding assay.

2.2. Isolation of rat 5-HT_3 receptor cDNA

Rat 5-HT_3 cDNA was amplified from random-primed cDNA of rat brain by PCR amplification. The forward primer S1 was designed on the basis of sequence adjacent to the translation initiation codon of mouse 5-HT_3 receptor cDNA, because the cDNA sequence corresponding to the putative signal peptide of rat 5-HT_3 receptor had not been published. The reverse primers S6 and S8 were based on the published sequence of rat 5-HT_3 receptor cDNA. The deduced amino acid sequence of an amplified cDNA (1.5 kbp) was identical to that reported by Isenberg et al. (1993), except that Arg283 (CGC) was replaced by Gly (GGC) in the mature polypeptide.

2.3. Expression and preparation of cloned rat 5-HT_3 receptor

A cDNA fragment containing the entire coding region of rat 5-HT_3 receptor (1.5 kbp) was subcloned into the mammalian expression vector pEF-BOS. COS-1 cells were transfected with the plasmid according to the DEAE-dextrane/chloroquine method (Luthman and Magnusson, 1983). COS-1 cells ($1–2 \times 10^6$ cells) were incubated overnight, exposed to the plasmid DNA (15 μg) with DEAE-dextrane (0.25 mg/ml) for 14 h and exposed to 0.1 mM chloroquine for 2.5 h. After 3 days' culture, the transfected cells were homogenized in 50 mM Hepes buffer, and centrifuged at $48\,000 \times g$ for 10 min. The pellet was resuspended in Hepes buffer and recentrifuged as above.

2.4. Radioligand binding assay

For saturation studies, membranes were incubated with increasing concentrations of [^{3}H]YM060 (0.01–0.2 nM) in a final volume of 0.5 ml for 30 min at 25°C. For competition studies, a single concentration of [^{3}H]YM 060 (0.03 nM) and 4–6 concentrations of agonists and antagonists were used. An incubation time of 10 min was employed for 5-HT and 2-methyl-5-HT, because 30 min incubation resulted in raised K_i values especially in rat intestine tissues. The incubation was terminated by a rapid filtration through Whatman GF/B filters using a Brandel cell harvester (Brandel, Gaithersburg, MD, USA), followed by washing of the filter 3 times with 3 ml of ice-cold Hepes buffer. Radioactivity retained on the filters was counted with a liquid scintillation counter (Packard 2000CA). Non-specific binding was determined in the presence of 1 μM of tropisetron. The protein content of each membrane suspension was measured by the method of Bradford (1976).

2.5. Analysis of data

Values were expressed as the mean ± S.E.M. Comparisons between values from different groups were evaluated by analysis of variance. Probabilities of $< 5\%$ ($P < 0.05$) were considered significant. IC_{50} values, the concentration required to inhibit specific binding by 50%, were calculated by logit-log analysis from the following equation: $\log[(B_0 - B_i)/(B_i - B_n)] = n[\log(\text{antagonist concentration}) - \log(IC_{50})]$ where B_0 and B_i are binding in the absence and presence of the antagonist to be tested, respectively; B_n is non-specific binding and n is the slope factor identical to the Hill coefficient. The inhibition constants (K_i values) were calculated from IC_{50} values using the following equation: $K_i = IC_{50}/(1 + [L]/K_d)$ where [L] is the radioligand concentration and K_d is the dissociation constant of the radioligand.

2.6. Drugs

[^{3}H]YM060 (78 Ci/mmol) was specially synthesized by Amersham International (Buckinghamshire, UK) for Yamanouchi Pharmaceutical Co. (Tsukuba, Japan). YM060 ((*R*)-5-[(1-methyl-1*H*-indol-3-yl)carbonyl]-4,5,6,7-tetrahydro-1*H*-benzimidazole monohydrochloride), YM114 (KAE-393, (*R*)-5-[(1-indolinyl) carbonyl]-4,5,6,7-tetrahydro-1*H*-benzimidazole monohydrochloride), ondansetron (1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1*H*-imidazole-1-yl)methyl]-4*H*-carbazole-4-one monohydrochloride), granisetron (BRL43694, endo-1-methyl-*N*-(9-methyl-azabicyclo[3.3.1]non-3-yl)-1*H*-indazole-3-carboxamide), tropisetron (ICS205-930, endo-8-methyl-8-azabicyclo[3.2.1]oct-3-yl-1*H*-indole-3-carboxylate), *m*-chlorophenylbiguanide and 2-methyl-5-HT were prepared by Yamanouchi Pharmaceutical Co. 5-HT creatinine sulfate was purchased from E. Merck (Darmstadt, Germany).

3. Results

3.1. Saturation analysis

Specific binding of [^{3}H]YM060 (0.01–0.2 nM, defined using 1 μM tropisetron) was detectable in each native tissue and cloned rat 5-HT$_3$ receptors. Scatchard analysis revealed that the binding was apparently to a single site with high affinity (Table 1).

There was no statistically significant difference in the affinity of [^{3}H]YM060 in the rat cerebral cortex (K_d = 8.4 ± 0.2 pM, n = 3), ileum (K_d = 9.8 ± 0.2 pM, n = 3) and colon (K_d = 6.4 ± 1.5 pM, n = 3). The affinity of [^{3}H]YM060 in cloned rat 5-HT$_3$ receptors (K_d = 21.2 ± 2.6 pM, n = 3) was similar to that obtained in the native tissues.

3.2. Competition analysis

A variety of 5-HT$_3$ receptor agonists and antagonists were tested for their ability to inhibit specific binding of [^{3}H]YM060 in the native and the cloned rat 5-HT$_3$ receptors. 5-HT$_3$ receptor agonists and antagonists dose dependently competed with [^{3}H]YM060 binding. pK_i values and Hill coefficients for a series of compounds are shown in Table 2.

The affinity of 5-HT$_3$ receptor agonists and antagonists was similar between the brain and the intestine. The rank order of affinities was the same in the three native tissues and the cloned 5-HT$_3$ receptors. Although the pK_i values of most compounds were lower in the cloned rat 5-HT$_3$ receptors than in the native tissues, the correlation coefficient of the affinities between the cloned rat 5-HT$_3$ receptors and the native tissues was significantly high (r = 0.93; cloned 5-HT$_3$ receptor vs. brain, r = 0.94; cloned 5-HT$_3$ receptor vs. ileum, r = 0.94; cloned 5-HT$_3$ receptor vs. colon).

Table 1
The dissociation constant (K_d) and binding density (B_{max}) for [^{3}H]YM060 binding in different membranes of rats

Membrane	K_d (pM)	B_{max} (fmol/mg protein)
Native		
Rat brain [a]	8.4 ± 0.2	37.0 ± 0.8
Rat ileum	9.8 ± 0.2	14.9 ± 0.1
Rat colon	6.4 ± 1.5	9.9 ± 0.8
Cloned		
Rat 5-HT$_3$ receptors expressed in COS-1 cells	21.2 ± 2.6	1102.5 ± 149.6

Each value represents the mean ± S.E.M. from three experiments in triplicate. [a] Data from Akuzawa et al. (1995).

4. Discussion

The present study pharmacologically investigated the intra-species difference in rat 5-HT$_3$ receptors and made the first direct comparison of the native and the cloned 5-HT$_3$ receptors. In addition to the affinity of [^{3}H]YM060, inhibition of 5-HT$_3$ receptor agonists and antagonists was examined.

In order to determine the existence of an intra-species difference in rat 5-HT$_3$ receptors, [^{3}H]YM060 binding was examined in membrane homogenates prepared from three different tissues taken from the same rats. [^{3}H]YM060 is a highly potent and selective 5-HT$_3$ receptor radioligand in the cerebral cortex of rats (Akuzawa et al., 1995). Specific binding was sufficiently high to allow characterization in the ileum and the colon. The affinity of [^{3}H]YM060 showed no significant difference both in the rat brain and the rat intestine (ileum and colon). 5-HT$_3$ receptor agonists and antagonists had similar affinities for the three tissues. We can find no strong evidence for intra-species difference of rat 5-HT$_3$ receptor. The conflicting results of Bonhaus et al. (1993) that YM060 and *m*-chlorophen-

Table 2
Binding affinities of 5-HT$_3$ receptor agonists and antagonists for [^{3}H]YM060 binding sites in different tissues of rats

Membrane	Rat brain		Rat ileum		Rat colon		Cloned rat 5-HT$_3$ receptors	
Compounds	pK_i (−log M)	n_H	pK_i (−log M)	n_H	pK_i (−log M)	n_H	pK_i (−log M)	n_H
Antagonists								
YM060	11.47 ± 0.16	1.13 ± 0.07	11.48 ± 0.12	0.70 ± 0.05	11.68 ± 0.04	0.55 ± 0.13	10.86 ± 0.16	0.96 ± 0.07
YM114	10.76 ± 0.19	0.98 ± 0.19	10.60 ± 0.21	0.90 ± 0.13	10.95 ± 0.33	1.01 ± 0.12	9.97 ± 0.02	0.81 ± 0.02
Tropisetron	9.74 ± 0.18	0.94 ± 0.22	9.66 ± 0.13	1.11 ± 0.16	9.32 ± 0.15	1.13 ± 0.34	8.82 ± 0.03	1.11 ± 0.08
Granisetron	9.55 ± 0.09	0.98 ± 0.09	9.55 ± 0.23	0.89 ± 0.19	9.72 ± 0.13	0.79 ± 0.20	8.97 ± 0.01	0.92 ± 0.04
Ondansetron	8.89 ± 0.09	1.12 ± 0.08	8.69 ± 0.16	1.03 ± 0.02	8.45 ± 0.13	1.00 ± 0.18	8.58 ± 0.17	0.96 ± 0.03
Agonists								
m-CPBG	9.22 ± 0.07	1.36 ± 0.21	9.29 ± 0.14	1.04 ± 0.27	9.43 ± 0.06	1.13 ± 0.09	9.07 ± 0.14	1.25 ± 0.04
5-HT	7.73 ± 0.11	1.06 ± 0.16	7.45 ± 0.03	1.02 ± 0.13	7.34 ± 0.05	0.97 ± 0.03	7.55 ± 0.17	0.99 ± 0.09
2-Methyl-5-HT	7.62 ± 0.14	1.17 ± 0.14	7.43 ± 0.02	0.97 ± 0.03	7.21 ± 0.05	0.99 ± 0.12	7.23 ± 0.24	1.51 ± 0.05

The relative affinities of 5-HT$_3$ receptor agonists and antagonists at [^{3}H]YM060 binding sites in native and cloned 5-HT$_3$ receptors of rats. Data yielded from inhibition curves which were best fitted by one-site models and form which pK_i values ($-\log_{10} K_i$) were obtained. n_H represents the Hill coefficient. Each value represents the mean ± S.E.M. from three experiments in triplicate.

ylbiguanide have different affinity in brain and ileum of mice may be attributed to the difference in species or in radioligands.

To compare the native and cloned 5-HT_3 receptors, radioligand binding assay was performed. Although the affinities of [^{3}H]YM060 and pK_i values of 5-HT_3 receptor agonists and antagonists in the cloned receptors were slightly lower than those in the native tissues, the rank order of the affinity against 5-HT_3 receptors (YM060 > YM114 > tropisetron > granisetron > *m*-chlorophenylbiguanide > ondansetron > 5-HT > 2-methyl-5-HT) showed an excellent correlation between the native and the cloned rat 5-HT_3 receptors. This result suggests that there is not a difference between the native and the cloned rat 5-HT_3 receptors. Based on the fact that the cloned 5-HT_3 receptors are homo-oligomers composed of 5-HT_3 receptor A subunits and accumulated evidence of other ligand-gated ion channels, the native rat 5-HT_3 receptor may be homo-oligomers. This may confirm that intra-species differences are not present, because mRNA of cloned rat 5-HT_3 receptor A subunit is detected both in rat brain and in rat intestine (Miyake et al., 1995). The same features may be predicted in mouse and in human, because the respective mRNA of 5-HT_3 receptor A subunit is detected both in brain and in intestine (Miyake et al., 1995). One explanation for the small difference in the affinities between the native and the cloned rat 5-HT_3 receptors may be due to an artifact of the cloned receptors.

In conclusion, intra-species difference in rat 5-HT_3 receptors was not observed. The characteristics of [^{3}H]YM060 binding were the same for rat brain and rat intestine. Furthermore, [^{3}H]YM060 binding was similar between the native and the cloned rat 5-HT_3 receptor. The native rat 5-HT_3 receptor may be homo-oligomers composed of 5-HT_3 receptor A subunits.

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