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Short Communication

Changes in Characteristics of the Specific Binding of [^{3}H]LY-278584, a 5-HT$_3$–Receptor Antagonist, on Differentiated NG108-15 Cells

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Abstract. We have reported previously that the concentration of intracellular Ca^{2+} evoked by serotonin (5-HT) was significantly augmented in differentiated NG108-15 (NG) cells treated with dibutyryl cAMP and the enhanced response occurred via 5-HT$_3$ receptors. We investigated changes in the characteristics for specific binding of [^{3}H]LY-278584 (a specific antagonist of the 5-HT$_3$ receptor) on membranes from differentiated NG cells. The results indicated that the K_d and B_{max} values for the specific binding to differentiated NG cells were significantly smaller and larger, respectively, than those for undifferentiated NG cells. The binding was significantly inhibited by 10 nM tropisetron, a specific 5-HT$_3$–receptor antagonist, but not by any other types of 5-HT–receptor antagonists. These results suggested that the enhanced response by 5-HT in differentiated NG cells was due to both qualitative and quantitative changes in the 5-HT$_3$ receptor.

Keywords: specific binding of LY-278584 (a selective blocker of 5-HT$_3$ receptor), up-regulation of 5-HT$_3$ receptor, differentiated NG108-15 cell

The NG108-15 (NG) cell line, formed through the fusion of a mouse neuroblastoma (N18TG-2) and a rat glioma (C6BU-1) cell line, has been used as a suitable model system to investigate the mechanisms of neuronal development and differentiation (1). We have previously reported that the increase in the concentration of intracellular free-Ca^{2+} ion ($[Ca^{2+}]_i$) induced by a high concentration of KCl in dibutyryl cAMP (Bt_2cAMP)-treated (differentiated) NG cells was mainly due to quantitative, not qualitative, up-regulation of L-type voltage-gated Ca^{2+} ion channels (VGCCs) (2 – 4). Furthermore, it has also been reported by Kawaguchi et al. (5) that the enhancement of TTX-sensitive Na^+ current density in differentiated NG cells is mainly due to an increase in the expression of TTX-sensitive voltage-gated Na^+ ion channel $Na_V1.7$.

Imanishi et al. (6) have observed that the response to serotonin (5-HT) was prominently observed in differentiated NG cells (which was almost completely inhibited by 3 nM tropisetron, a 5-HT$_3$–receptor antagonist), but not in undifferentiated NG cells. The results suggested that there are some mechanisms by which the response evoked by 5-HT is up-regulated in differentiated NG cells. Recently, Imanishi et al. (7) have also reported that the 5-HT–induced Ca^{2+} response in differentiated NG cells was mainly due to L-type VGCCs allowing extracellular Na^+ to enter via 5-HT$_3$ receptors, but not through voltage-gated Na^+ ion channels.

It is known that 1-methyl-*N*-(8-methyl-8-azabicyclo(3.2.1)oct-3-yl)-1*H*-indazole-3-carboxamide (LY-278584), which has a very similar structure to granisetron, is a specific antagonist for 5-HT$_3$ receptors in rat (8, 9) and human brain (10) and that its radiolabeled congener, [^{3}H]LY-278584, binds to 5-HT$_3$ receptors in rat and human brain with high affinity. As this ligand exhibited a very good selectivity and specificity in membranes from rat and human brain, it appeared to be the best ligand to choose for studying 5-HT$_3$ sites in NG cells. Therefore, we investigated whether there was any change in characteristics for the specific binding of [^{3}H]LY-278584 in differentiated NG cells to better understand the mechanism for the up-regulated response by 5-HT.

NG cells (passage number <15) were grown by a modification of the method of Ichida et al. (2) and Imanishi et al. (3). NG cells were harvested and subcultured by re-seeding at a density of 10^2 – 10^3 cells/mm^2 in

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100-mm culture dishes that were precoated with poly-L-ornithine. The following day, 0.75 mM Bt_2cAMP was added to the cultures to induce cell differentiation. The supplemented medium was replaced every 2 days, and the NG cells were used for the experiments on day 6 after the addition of 0.75 mM Bt_2cAMP, when the up-regulated response by 5-HT has reached a plateau (2). Undifferentiated NG cells were grown under the same conditions with the medium devoid of Bt_2cAMP.

Crude membranes from differentiated and undifferentiated NG cells were prepared by a modification of the method of Ichida et al. (11). NG cells were detached from dishes and the cell suspensions were centrifuged at 130 × *g* for 10 min. The pellet was suspended in homogenization solution [20 mM 4-(2-hydroxyethyl)-l-piperazine ethanesulfonic acid (HEPES)-NaOH (pH 7.4 at 4°C), 1 mM benzamidine, 10 μg/ml antipain, 10 μg/ml chymostatin, 10 μg/ml leupeptin, and 5 μg/ml pepstatin A]. The homogenate was centrifuged at 1,000 × *g* for 10 min. Then, the supernatant was centrifuged at 40,000 × *g* for 20 min. This procedure was repeated and the final pellets were suspended in 10 mM HEPES-NaOH (pH 7.4 at 4°C).

The specific [^{3}H]LY-278584 binding to crude membranes from differentiated and undifferentiated NG cells was assayed by a modification of the methods of Ichida et al. (11) and Delgado et al. (12). A 25-μl aliquot of the crude membranes (about 25 μg of protein/tube) were added to assay medium consisting of 30 mM HEPES-NaOH (pH 7.4 at 4°C), 0.15M NaCl, 1 mM $CaCl_2$, 10 μM pargyline, 0.1% ascorbic acid, and various concentrations of [^{3}H]LY-278584 with or without tropisetron at 1,000-fold the concentration of [^{3}H]LY-278584. The final volume of the assay system was 300 μl. After incubation for 60 min at 30°C, the mixture was rapidly filtered through a Whatman GF/F glass filter that had been preimmersed in ice-cold washing solution consisting of 30 mM HEPES-NaOH (pH 7.4 at 4°C) with suction, and then the filter was washed three times with 2 ml of ice-cold washing solution. The radioactivity on the filter was counted in a liquid scintillation counter.

Statistical analyses were performed by the paired or unpaired Student's *t*-test and multiple groups were evaluated by one-way analysis of variance. $P < 0.05$ was considered statistically significant.

Bt_2cAMP, cyanopindolol, ketanserin, mianserin, poly-L-ornithine, protease inhibitors, ritanserin, SB204070, thymidine, and tropisetron were purchased from Fluka Chemie AG (Buchs, Switzerland), Kyowa Hakko Kirin Co. (Tokyo), and Sigma (St. Louis, MO, USA). [^{3}H]LY-278584 was purchased from Amersham Biosciences Co. (Tokyo).

The specific [^{3}H]LY-278584 binding to crude membranes from differentiated and undifferentiated NG cells as a function of the concentration of [^{3}H]LY-278584 are shown in Fig. 1A. The specific binding of [^{3}H]LY-278584 to the crude membranes were saturable, and the concentrations for half-maximal saturation in crude membranes from Bt_2cAMP-treated and untreated NG cells were about 0.8 (n = 5) and 2.6 (n = 6) nM, respectively. At each concentration of [^{3}H]LY-278584 tested, the amount of specific [^{3}H]LY-278584 binding to crude membranes from differentiated NG cells was significantly higher than that from undifferentiated NG cells.

Scatchard and Hill plots for the specific binding of [^{3}H]LY-278584 to the crude membranes are shown in Fig. 1. The K_d and B_{max} values calculated from the Scatchard plots were 0.65 ± 0.18 nM and 3.12 ± 0.78 pmol/mg of protein in differentiated NG cells (n = 4 – 6), respectively, and 1.42 ± 0.04 nM and 1.78 ± 0.02 pmol/mg of protein in undifferentiated NG cells (n = 4 – 6), respectively. The K_d and B_{max} values for the specific binding to differentiated NG cells were significantly smaller and larger, respectively, than those for undifferentiated NG cells, suggesting that the specific binding sites of [^{3}H]LY-278584 (which must be 5-HT_3 receptors) on differentiated NG cells might result in both qualitative and quantitative changes. The Hill coefficient for the specific binding to differentiated and undifferentiated NG cells was 1.030 ± 0.06 and 1.019 ± 0.08 (n = 4 – 6), respectively, suggesting that specific [^{3}H]LY-278584 binding sites on crude membranes from differentiated and undifferentiated NG cells do not show negative or positive cooperativity.

The specific [^{3}H]LY-278584 binding to crude membranes from differentiated and undifferentiated NG cells was significantly inhibited (about 80% inhibition) by 10 nM tropisetron, a specific 5-HT_3–receptor antagonist, but not by any other types of 5-HT–receptor antagonists such as cyanopindolol (5-HT_{1A} and $_{1B}$), ketanserin (5-HT_2), mianserin (5-HT_{1D} and $_2$), ritanserin (5-HT_{1C} and $_{2A}$), and SB204070 (5-HT_4) at the concentration tested (10 nM) (Fig. 2A). These results suggest that the binding sites on crude membranes from differentiated and undifferentiated NG cells are mainly composed of 5-HT_3 receptors.

The inhibitory effect of tropisetron on the specific [^{3}H]LY-278584 binding to crude membranes from differentiated and undifferentiated NG cells was dose-dependent (Fig. 2B). The IC_{50} value of tropisetron for the specific binding to differentiated and undifferentiated NG cells was 1.33 ± 0.02 and 1.95 ± 0.01 (n = 6 – 8) nM, respectively, indicating that there is no change in the affinity of tropisetron binding sites on differentiated and undifferentiated NG cells.

In this work, we have investigated the characteristics of specific [^{3}H]LY-278584 binding to crude membranes

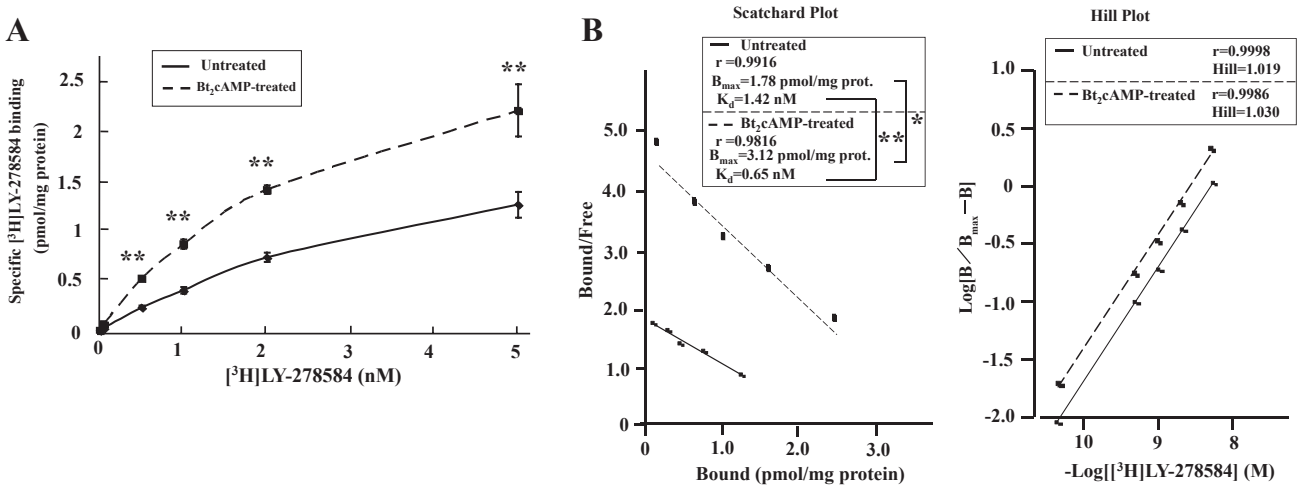

Fig. 1. Specific [^{3}H]LY-278584 binding as a function of [^{3}H]LY-278584 concentration (A) and Scatchard and Hill plots (B). A: $^{**}P < 0.01$ vs. specific [^{3}H]LY-278584 binding at each concentration of [^{3}H]LY-278584 to crude membranes from undifferentiated NG cells. Points are the means of the data from 4 to 6 independent experiments. Bars indicate standard errors. B: Points are the means of the data from 4 to 6 independent experiments.

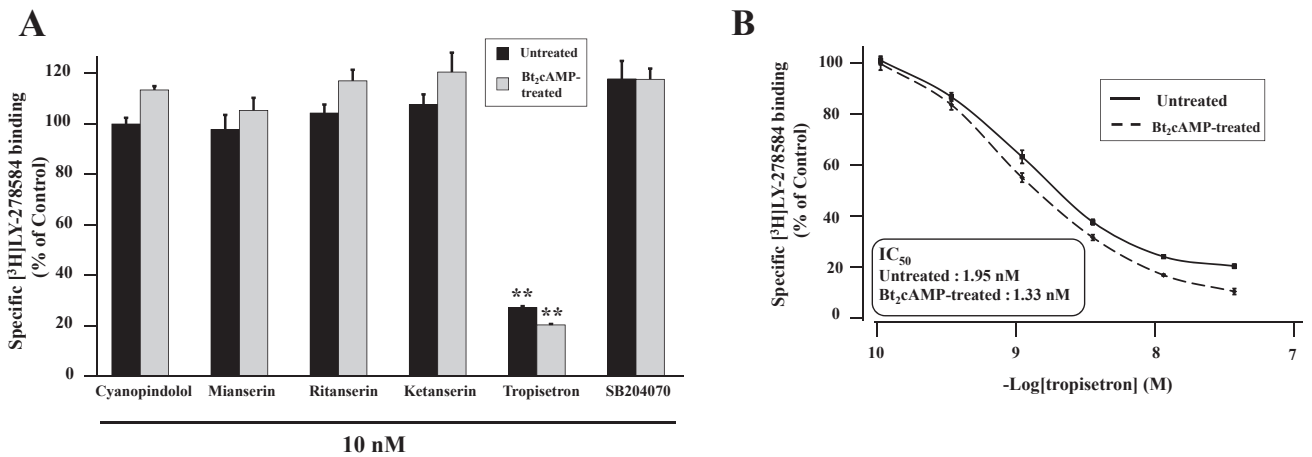

Fig. 2. Effect of various 5-HT–receptor antagonists (A) and inhibitory effects of various concentrations of tropisetron (B) on specific [^{3}H]LY-278584 binding. A: $^{**}P < 0.01$ vs. specific [^{3}H]LY-278584 binding in the control (without blockers) for differentiated or undifferentiated NG cells. Columns are the means of the data from 6 independent experiments. Bars indicate standard errors. B: Points are the means of the data from 6 to 8 independent experiments. Bars indicate standard errors.

from Bt$_2$cAMP-treated and untreated NG cells. LY-278584 was used as a specific antagonist for 5-HT$_3$ receptors in rat (8, 9) and human brain (10).

The radiolabeled congener [^{3}H]LY-278584 specifically bound to 5-HT$_3$ receptors in rat and human brain with high affinity; the K_d value in rat (9) and human brain (10) was 1.5 and 3.08 nM, respectively. These K_d values were comparable with the K_d values obtained using NG cells in this work, 0.65 (differentiated) and 1.42 (undifferentiated) nM, as shown in Fig. 1B. Furthermore, the results indicated that the specific [^{3}H]LY-278584 binding sites on crude membranes from differentiated and undifferentiated NG cells are mainly 5-HT$_3$ receptors because the binding to crude membranes from these NG cells was significantly inhibited by about 80% by 10 nM tropisetron, but not by any other types of 5-HT–receptor antagonists (such as 5-HT$_{1A, 1B, 1C, 1D, 2,}$ and $_{2A}$ receptors) at the same concentration of 10 nM (Fig. 2A). Taken together, it seems likely that radiolabeled [^{3}H]LY-278584 binds specifically to 5-HT$_3$ receptors in differentiated and undifferentiated NG cells.

The K_d and B_{max} values for specific [^{3}H]LY-278584

binding to differentiated NG cells were significantly smaller and larger, respectively, than those to undifferentiated NG cells, suggesting that the 5-HT_3 receptors on differentiated NG cells might undergo both qualitative and quantitative changes compared with undifferentiated NG cells (Fig. 1: A and B). So far, five 5-HT_3 subunits have been cloned, 5-HT_{3A} – 5-HT_{3E} (13, 14). Whereas homomeric 5-HT_{3A} receptors are functional, the other subunits only form functional receptors when coexpressed with 5-HT_{3A} (13, 14). In addition, there have been some reports that the function of 5-HT_3 receptors depend on 5-HT_{3B} subunits in conjunction with 5-HT_{3A} subunits (15). Function may be altered by changing the ratio of 5-$HT_{3A/3B}$ subunits (13). While, Emerit et al. (16) have reported that although the total amount of 5-HT_3 receptor mRNA decreased in the differentiated NG cells by Bt_2cAMP, the ratio of two splice variants of the 5-HT_3 receptor (5-HT_3-R-A_S mRNA / 5-HT_3-R-A_L mRNA) increased about 3.3-fold. The second intracellular loop of 5-HT_3-R-A_L, and not 5-HT_3-R-A_S, has been shown to include a serine residue that potentially controls receptor desensitization by being phosphorylated. Taken together, these results indicate the possibility that the up-regulated response via 5-HT_3 receptors in differentiated NG cells might be due to relative changes in the proportion of 5-HT_{3A} and 5-HT_{3B} receptors or in that of 5-HT_3-R-A_S and 5-HT_3-R-A_L.

The 5-HT_3 receptor has been implicated not only in the pre- and post-synaptic regulation of several neurotransmitter pathways in the mammalian CNS, but also in mechanisms of nociception in the PNS. Therefore, to understand the mechanisms of the 5-HT_3 receptor expression, it would be very important to examine the time-dependent changes in mRNA and protein levels for 5-$HT_{3A/3B}$ subunits or for 5-HT_3-R-A_S / 5-HT_3-R-A_L in Bt_2cAMP-treated (differentiated) NG cells.

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