Regular Article

Effect of Selective Serotonin (5-HT)$_{2B}$ Receptor Agonist BW723C86 on Epidermal Growth Factor/Transforming Growth Factor-*α* Receptor Tyrosine Kinase and Ribosomal p70 S6 Kinase Activities in Primary Cultures of Adult Rat Hepatocytes

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Serotonin (5-hydroxytryptamine; 5-HT) can induce hepatocyte DNA synthesis and proliferation by autocrine secretion of transforming growth factor (TGF)-*α* through 5-HT$_{2B}$ receptor/phospholipase C (PLC)/Ca^{2+} and a signaling pathway involving epidermal growth factor (EGF)/TGF-*α* receptor tyrosine kinase (RTK)/extracellular signal-regulated kinase 2 (ERK2)/mammalian target of rapamycin (mTOR). In the present study, we investigated whether 5-HT or a selective 5-HT$_{2B}$ receptor agonist BW723C86, would stimulate phosphorylation of TGF-*α* RTK and ribosomal p70 S6 kinase (p70S6K) in primary cultures of adult rat hepatocytes. Western blotting analysis was used to detect 5-HT- or BW723C86 (10^{-6} M)-induced phosphorylation of EGF/TGF-*α* RTK and p70S6K. Our results showed that 5-HT- or BW723C86 (10^{-6} M)-induced phosphorylation of EGF/TGF-*α* RTK peaked at between 5 and 10 min. On the other hand, 5-HT- or BW723C86 (10^{-6} M)-induced phosphorylation of p70S6K peaked at about 30 min. Furthermore, a selective 5-HT$_{2B}$ receptor antagonist LY272015, a specific PLC inhibitor U-73122, a membrane-permeable Ca^{2+} chelator BAPTA/AM, an L-type Ca^{2+} channel blocker verapamil, somatostatin, and a specific p70S6K inhibitor LY2584702 completely abolished the phosphorylation of p70S6K induced by both 5-HT and BW723C86. These results indicate that phosphorylation of p70S6K is dependent on the 5-HT$_{2B}$-receptor-mediated autocrine secretion of TGF-*α*. In addition, these results demonstrate that the hepatocyte proliferating action of 5-HT and BW723C86 are mediated by phosphorylation of p70S6K, a downstream element of the EGF/TGF-*α* RTK signaling pathway.

Key words serotonin; signal transduction; ribosomal p70 S6 kinase (p70S6K); transforming growth factor-*α*; proliferation (cultured hepatocyte)

INTRODUCTION

Although active growth in mature rat hepatocytes is rare, hepatocytes sometimes proliferate to recover liver function rapidly after liver injury, for example, after 70% partial hepatectomy; this phenomenon is known as liver regeneration, and it is a process in which numerous cytokines, hormones, and growth factors are essential.[1,2] Platelet-derived serotonin (5-hydroxytryptamine; 5-HT) is also reported to be involved in liver regeneration.[3,4]

5-HT is known as a neurotransmitter with various functions, such as vascular constriction and platelet aggregation. It is also associated with the proliferation of some cell lines.[5,6] In addition, 5-HT has been associated with osteoblast, fibroblast, and pancreatic beta cell proliferation.[7–9] As for the liver, there are some reports that treatment with 5-HT and epidermal growth factor (EGF) enhances hepatocyte proliferation in primary culture and promotes liver regeneration *in vivo* after 70% partial hepatectomy.[10,11]

Previously, we demonstrated that 5-HT and a selective 5-HT$_{2B}$ receptor agonist BW723C86,[12] induce DNA synthesis and proliferation and increase EGF/transforming growth factor (TGF)-*α* receptor tyrosine kinase (RTK) phosphorylation and extracellular signal-regulated kinase 2 (ERK2) phosphorylation in primary cultures of rat adult hepatocytes.[13] In

these hepatocytes, the proliferative effect induced by 5-HT or BW723C86 is mediated through the activation of a signaling pathway involving 5-HT$_{2B}$ receptor/Gq/phospholipase C (PLC)/Ca^{2+} *via* autocrine secretion of TGF-*α*.[14] TGF-*α* secreted by cultured hepatocytes accelerates DNA synthesis and proliferation *via* activation of a signaling pathway involving EGF/TGF-*α* RTK, phosphatidylinositol 3-kinase (PI3K), ERK2, and mammalian target of rapamycin (mTOR). Proliferating signals by TGF-*α* may be transmitted to a downstream signaling element, mTOR and ribosomal p70 S6 kinase (p70S6K).[15,16] However, whether 5-HT or BW723C86 actually stimulates p70S6K activities and the order of activation downstream of TGF-*α* receptor in primary cultures of adult rat hepatocytes remains unknown. Therefore, to elucidate the signal transduction pathway, in the present study, we compared the time courses between the 5-HT- or BW723C86-induced phosphorylation of EGF/TGF-*α* RTK and that of p70S6K and investigated whether p70S6K is phosphorylated by 5-HT$_{2B}$ receptor/PLC/Ca^{2+} and EGF/TGF-*α* RTK/PI3K/ERK2/mTOR pathways using specific stimulators or inhibitors of signal transducer.

MATERIALS AND METHODS

Animals Male Wistar rats obtained from Tokyo Experi-

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mental Animal Co. (Tokyo, Japan) were used in the present study as previously descried.[13] All rats were treated in compliance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society and Josai University.

Hepatocyte Isolation and Culture Normal hepatocytes were isolated using the two-step _in situ_ collagenase perfusion technique described by modified Seglen[17] to facilitate disaggregation of the adult rat liver.[18] All hepatocytes showed a viability of >97% as determined by Trypan blue exclusion. Isolated hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 onto plastic and collagen-coated 33-mm culture dishes (Iwaki Glass Co., Tokyo, Japan) and were cultured, as previously study.[13]

The medium was then removed by aspiration and the cells were cultured in dexamethasone- and serum-free Williams' medium E supplemented with 5-HT or BW723C86. Finally, the following were added as appropriate: 5-HT or BW723C86 with or without selective 5-HT$_{2A}$ (ketanserin), 5-HT$_{2B}$ (LY272015), and 5-HT$_{2C}$ receptor antagonist (SB242084), U-73122, U-73343, GF109203X, H-89, 2,4-dideoxyadenosine, BAPTA/AM, verapamil, somatostatin, monoclonal antibody against TGF-α and growth-related signal transducer inhibitor (AG1478, LY294002, PD98059, rapamycin, and LY2584702).

Determination of RTK Activity Immunoblotting with corresponding anti-phospho-RTK was performed in accordance with the manufacturer's instructions to determine RTK activity, as previously described[19]; as a result, a 175-kDa protein was identified as the EGF/TGF-α receptor. Briefly, hepatocytes were isolated and seeded at a cell density of 3.3×10^4 cells/cm^2 and cultured in Williams' medium E containing 5% newborn bovine serum for 3h. The cultured hepatocytes were then washed with ice-cold phosphate-buffered saline (PBS) (pH 7.4), and 0.2mL lysis buffer (20mM Tris–HCl buffer, pH 7.5, 1% Triton X-100, 150mM NaCl, 1mM ethylendiamine-N,N,N',N'-tetraacetic acid, 1mM ethylene glycol bis(2-aminoethlether)-N,N,N',N'-tetraacetic acid (EGTA), 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM phenylmethylsulfonyl fluoride) was added. Cell lysates were obtained by scraping the cells in the lysis buffer followed by sonication for 3min. Cell lysates were then spun down ($3000 \times \boldsymbol{g}$ for 3min at 4°C), and the supernatants were denatured in boiling water for 5min. For immunoblotting analysis using anti-phospho-EGF/TGF-α receptor (Tyr1068) antibody, samples of the supernatant ($30\,\mu$g/lane) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% acrylamide resolving gel, according to Laemmli's method.[20] Following the electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane and immunoblotted with anti-phospho-tyrosine antibodies. Following incubation with horseradish peroxidase-conjugated secondary antibodies, blots were visualized using enhanced chemiluminescence reagents (PerkinElmer, Inc., Waltham, MA, U.S.A.). After the membrane was visualized using the enhanced chemiluminescence reagent and exposed to Hyperfilm (Kodak, Tokyo, Japan), densitometric analysis was performed to quantify the proteins using the National Institutes of Health image program (ver. 1.6 for Macintosh). Next, the phosphorylated tyrosine kinase activity of the p175-kDa protein was normalized to that of the total p175-kDa protein. Finally, to determine the supernatant protein concentration, a modified Lowry procedure was carried out using bovine serum albumin (BSA) as a standard.[21]

Determination of p70S6K Activity Phosphorylated p70S6K was identified by Western blotting analysis using anti-phospho-p70S6K monoclonal antibody according to the manufacturer's instructions. Briefly, the treated cells were washed with ice-cold PBS (pH 7.4) and lysed with cell lysis buffer (10mM Tris–HCl buffer, pH 7.4, 0.1% SDS, 150mM NaCl, 1mM EGTA, 2mM dithiothreitol, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, $10\,\mu$g/mL leupeptin, and $10\,\mu$g/mL aprotinin), after which, the hepatocytes were harvested followed by sonication for 3min. The cell lysates were then spun down ($14000 \times \boldsymbol{g}$ for 35min at 4°C) and the supernatants denatured in boiling water for 5min. Next, Western blotting procedures were carried out as described in the previous section, and in accordance with Laemmli's method, applications of $20\,\mu$g of supernatant per lane of 10% polyacrylamide resolving gel were excluded.[20] Phosphorylated p70S6K activity was then normalized to the total p70S6K activity. Finally, to quantify the cytosolic protein concentration in the hepatocytes, a modified Lowry procedure was carried out using BSA as a standard.[21]

Determination of Cell Proliferation The number of nuclei was determined as previously described.[13] Nuclei rather than cells were counted to assess proliferation.[15]

Materials Regarding the selective antagonists for 5-HT$_2$ receptor subtypes, ketanserin tartrate was obtained from Enzo Life Sciences (Farmingdale, NY, U.S.A.), LY272015 hydrochloride was purchased from Santa Cruz Biotechnology (Dallas, TX, U.S.A.), and SB204084 was obtained from Tocris Bioscience (Bristol, U.K.) In addition, 5-HT, dexamethasone, somatostatin, verapamil hydrochloride, and aprotinin were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). BAPTA/AM [1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)] was purchased from Santa Cruz Biotechnology. Rapamycin, U-73122 (1-[6-[[17β-3-methoxyestra1-,3,5(10)-trien-17-yl]-amino]hexyl]-1H-pyrrol-2,5-dione), U-73343 (1-[6-[[17β-3-metoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidine), GF109203X (2-[1-(3-dimethyaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide), 2,4-dideoxyadenosine, H-89 (N-[2-(p-bromo-cinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride), AG1478 (2-[4-morpholinyl]-8-phenyl-1(4H)-benzopyran-4-one), and LY294002 (N-[3-chorophenyl]-6,7-dimethoxy-4-quinazolinamine) were from purchased from Enzo Life Sciences. LY2584702 (4-[4-[4-[4-fluoro-3-(trifluoromethyl)phenyl]-1-methyl-1H-imidazol-2-yl]-1-piperidinyl]-1H-pyrazol[3,4-d]pyrimidine was obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). PD98059 (2′-amino-3′-methoxyflavone) was purchased from Calbiochem-Behring (La Jolla, CA, U.S.A.), Williams' medium E containing 5% newborn calf serum was obtained from Flow Laboratories (Irvine, Scotland), and collagenase (type II) was purchased from Worthington Biochemical (Freehold, NJ, U.S.A.). The monoclonal antibody to TGF-α (sc-374433) was purchased from Santa Cruz Biotechnology, anti-phospho-EGF/TGF-α receptor (Tyr1068) antibody (ab40815) was purchased from Abcam Inc. (Cambridge, U.K.), and anti-phospho-p70S6K monoclonal antibody (#9234) was purchased from Cell Signaling Technology (Beverly, MA, U.S.A.); all other reagents were of analytical grade.

Data Analysis and Statistics Data were expressed as the mean ± standard error of the mean (S.E.M.). ANOVA for unpaired data followed by *post-hoc* analysis with Dunnett's multiple comparison test were used for group comparisons, and p values <0.05 were considered to indicate statistical significance.

RESULTS

Time Course of 5-HT- or BW723C86-Induced Phosphorylation of EGF/TGF-α RTK (p175 kDa) The effects of 5-HT- or BW723C86-induced phosphorylation of EGF/TGF-α RTK were investigated and compared. Figure 1A shows the typical pattern of phospho-EGF/TGF-α RTK (p175 kDa) in isolated hepatocytes in culture as detected by Western blotting analysis. The phosphorylated EGF/TGF-α RTK band was induced after 1 min, with the peak, which was about a threefold increase compared with control, occurred at between 5 and 10 min. Within 60 min after the addition of 5-HT (10^{-6} M) or BW723C86 (10^{-6} M), this EGF/TGF-α RTK phosphorylation rapidly decreased to baseline levels (Figs. 1A, B). EGF/TGF-α RTK phosphorylation was not significantly affected by medium alone (control) at any time during treatment (Figs. 1A, B).

Time Course of 5-HT- or BW723C86-Induced Phosphorylation of p70S6K The time courses of 5-HT- and BW723C86-induced phosphorylation of p70S6K were then examined and compared. Figure 2 shows the typical pattern of phospho-p70S6K in isolated hepatocytes in culture as detected by Western blotting analysis. The phosphorylated p70S6K band was significantly induced at about 30 min (about a 1.8-fold increase). After the addition of 5-HT or BW723C86, the phosphorylation of p70S6K slowly decreased until 60 min (Figs. 2A, B); it was not significantly affected by medium alone (control) at any time during treatment (Figs. 2A, B).

Effects of Selective Antagonists of 5-HT$_2$ Receptor Subtypes or Specific Inhibitors of Growth-Related Signal Transducers on Phosphorylation of p70S6K Induced by 5-HT or BW723C86 The effects of selective antagonists of 5-HT$_{2B}$ receptor subtypes or specific inhibitors of growth-related signal transducers on 5-HT- or BW723C86-induced phosphorylation of p70S6K were then examined at 30 min after addition. As shown in Fig. 3, 5-HT- or BW723C86-induced phosphorylation of p70S6K is completely blocked by LY272015 (10^{-6} M), the 5-HT$_{2B}$ receptor antagonist,[22] but not ketanserin (10^{-6} M), the 5-HT$_{2A}$ receptor antagonist,[23] or SB242084 (10^{-6} M), the 5-HT$_{2C}$ receptor antagonist.[24] Moreover, 5-HT- or BW723C86-induced phosphorylation of p70S6K was abolished by the EGF RTK inhibitor AG1478 (10^{-6} M),[25] the PI3K inhibitor LY294002 (3×10^{-7} M),[26] the mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor PD98059 (10^{-6} M),[27] the mTOR inhibitor rapamycin (10 ng/mL),[28] and the p70S6K inhibitor LY2584702 (10^{-8} M).[29]

Dose-Dependent Effects of Specific Inhibitor of p70S6K on Hepatocyte Proliferation Induced by 5-HT or BW723C86 We then examined the dose-dependent effects of specific inhibitor of p70S6K LY2584702 (10^{-10} to 10^{-7} M)[29] on proliferation in primary cultured hepatocytes induced by 5-HT (10^{-6} M) or BW723C86 (10^{-6} M). As shown in Fig. 4, mitogenesis in cultured hepatocytes induced by 5-HT or BW723C86 was blocked by LY2584702 in a dose-dependent manner after culturing for 4 h. The maximal inhibition of hepatocyte proliferation occurred at 10^{-8}–10^{-7} M, respectively. These results support the hypothesis that 5-HT- or BW723C86-induced phosphorylation of p70S6K is mediated through the activation of the 5-HT$_{2B}$ receptor and a signaling pathway involving EGF/TGF-α RTK/PI3K/MEK/mTOR.

Effects of Specific Signal Transduction Inhibitors Downstream of G Protein-Coupled Receptors on p70S6K Phosphorylation Induced by 5-HT or BW723C86 To characterize the involvement of 5-HT- or BW723C86-induced
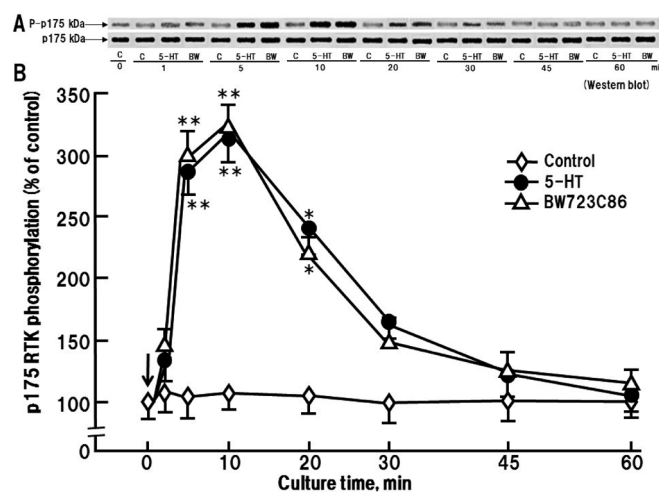

Fig. 1. Time Course of EGF/TGF-α Receptor Tyrosine Kinase (RTK) (p175 kDa) Phosphorylation Stimulated by 5-HT or BW723C86

Hepatocytes were plated at a cell density of 3.3×10^4 cells/cm^2 and cultured for 3 h. Next, the medium was rapidly replaced with serum-free Williams' medium E, and the hepatocytes were cultured with 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) for various times. Determination of phosphorylated EGF/TGF-α RTK induced by 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) is described in Materials and Methods. (A) Western blot image; (B) EGF/TGF-α RTK phosphorylation. Results are expressed as a percentage of the respective control value (mean ± S.E.M. of three separate experiments). * $p < 0.05$, ** $p < 0.01$ compared with control.
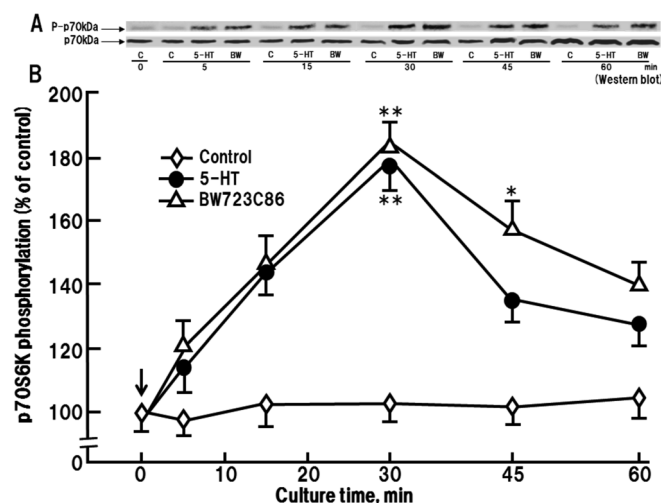

Fig. 2. Time Course of p70S6K Phosphorylation Stimulated by 5-HT or BW723C86

Hepatocytes were plated at a cell density of 3.3×10^4 cells/cm^2 and cultured for 3 h. Next, the medium was rapidly replaced with serum-free Williams' medium E, and the hepatocytes were cultured with 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) for various times. Determination of phosphorylated of p70S6K induced by 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) is described in Materials and Methods. (A) Western blot image; (B) p70S6K phosphorylation. Results are expressed as a percentage of the respective control value (mean ± S.E.M. of three separate experiments). * $p < 0.05$, ** $p < 0.01$ compared with control.
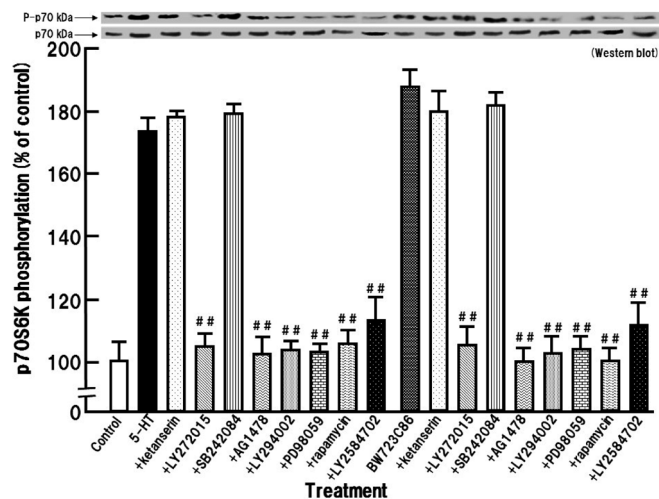
Fig. 3. Effects of Selective Antagonists of 5-HT$_2$ Receptor Subtypes or Specific Inhibitors of Growth-Related Signal Transducers on 5-HT- or BW723C86-Induced Phosphorylation of p70S6K

Hepatocytes were plated at a cell density of 3.3×10^4 cells/cm^2 and cultured for 3 h. Next, the medium was rapidly replaced with serum-free Williams' medium E, and the hepatocytes were cultured with 5-HT (10^{-6}M) or BW723C86 (10^{-6}M) with or without selective antagonists of 5-HT$_2$ receptor subtypes or specific inhibitors of growth-related signal transducers for 30 min. Determination of phosphorylated p70S6K stimulated by 5-HT or BW723C86 is described in Materials and Methods. Western blot analysis is shown at the top of each panel. Concentrations of agents used were as follows: ketanserin (10^{-6}M), LY272015 (10^{-7}M), SB242084 (10^{-6}M), AG1478 (10^{-6}M), LY294002 (3×10^{-7}M), PD98059 (10^{-6}M), rapamycin (10 ng/mL), and LY2584702 (10^{-8}M). Results are expressed as a percentage of the respective control value (mean ± S.E.M. of three separate experiments). [##]$p < 0.01$ compared with 5-HT or BW723C86.

Fig. 5. Effects of Specific Signal Transduction Inhibitors Downstream of G Protein-Coupled Receptors on 5-HT or BW723C86-Induced Phosphorylation of p70S6K

Hepatocytes were plated at a cell density of 3.3×10^4 cells/cm^2 and cultured for 3 h. Next, the medium was rapidly replaced with serum-free Williams' medium E, and the hepatocytes were cultured with 5-HT (10^{-6}M) or BW723C86 (10^{-6}M) with or without specific signal transduction inhibitors downstream of G protein-coupled receptors for 30 min. Determination of phosphorylated p70S6K stimulated by 5-HT or BW723C86 is described in Materials and Methods. Western blot analysis is shown at the top of each panel. Concentrations of agents used were as follows: U-73122 (10^{-6}M), U-73343 (10^{-6}M), H-89 (10^{-6}M), dideoxyadenosine (10^{-6}M), GF109209X (10^{-6}M), BAPTA/AM (10^{-7}M), verapamil (10^{-6}M), somatostatin (10^{-7}M), and mAb TGF-α (100 ng/mL). Results are expressed as a percentage of the respective control value (mean ± S.E.M. of three separate experiments). [##]$p < 0.01$ compared with 5-HT or BW723C86.
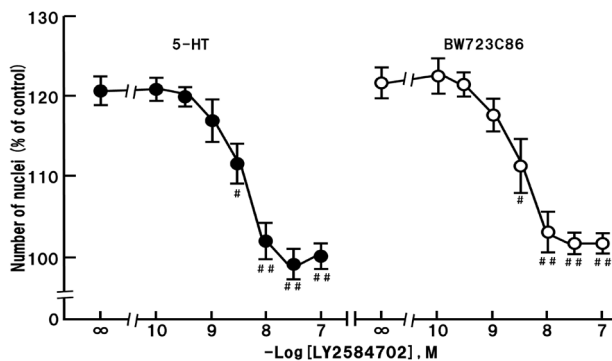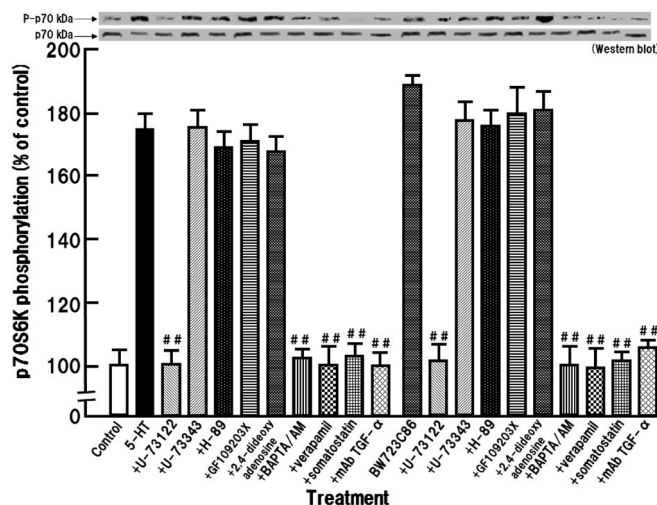

Fig. 4. Dose-Dependent Effects of Specific Inhibitor of p70S6K LY2584702 on Hepatocyte Proliferation Induced by 5-HT or BW723C86

Hepatocytes at a cell density of 3.3×10^4 cells/cm^2 were plated and cultured for 3 h. After changing the medium, the cultured hepatocytes were treated with increasing concentrations of LY2584702 (10^{-10} to 10^{-7}M) in the presence of 5-HT (10^{-6}M) or BW723C86 (10^{-6}M) for 4 h. Cell proliferation was assessed as previously descried. Results are expressed as the mean ± S.E.M. of three separate experiments. [#]$p < 0.05$, [##]$p < 0.01$ compared with 5-HT or BW723C86.

phosphorylation of p70S6K, we investigated the effects of specific signal transduction inhibitors downstream of G protein-coupled receptors. As shown in Fig. 5, although p70S6K phosphorylation was significantly induced by 5-HT or BW723C86 at 30 min, it was almost completely blocked by a PLC inhibitor U-73122 (10^{-6}M),[30] a membrane-permeable Ca^{2+} chelator BAPTA/AM (10^{-7}M),[31] an L-type Ca^{2+} channel blocker verapamil (10^{-6}M), somatostatin (10^{-7}M),[32] and the monoclonal antibody against TGF-α (100 ng/mL). These results suggested that 5-HT- or BW723C86-induced phosphorylation of p70S6K was mediated through the activation of the 5-HT$_{2B}$ receptor/Gq/PLC/Ca^{2+} signaling pathway and autocrine secretion of

TGF-α in hepatocytes. On the other hand, p70S6K phosphorylation induced by 5-HT or BW723C86 was not affected by an inactive structural analogue of U-73122 U-73343 (10^{-6}M), a protein kinase A inhibitor H-89 (10^{-6}M),[33] a protein kinase C inhibitor GF109203X (10^{-6}M),[34] or a direct adenylate cyclase inhibitor 2,4-dideoxyadenosine (10^{-6}M).[35] The proposed mechanism for the induction of hepatocyte DNA synthesis and proliferation *via* the 5-HT$_{2B}$ receptor is shown in a schematic in Fig. 6.

DISCUSSION

In a previous study, we suggested that stimulation of the 5-HT$_{2B}$ receptor subtype induces DNA synthesis and proliferation in primary cultures of rat adult hepatocytes.[13] Moreover, the proliferative effect induced by 5-HT or BW723C86 is mediated *via* autocrine secretion of TGF-α in hepatocytes through the activation of the 5-HT$_{2B}$ receptor/Gq/PLC/Ca^{2+} signaling pathway.[14] TGF-α is known as a primary mitogen that induces hepatocyte DNA synthesis and proliferation *via* activation of the EGF/TGF-α RTK/PI3K/ERK2/mTOR pathway; therefore, 5-HT is an indirect mitogen. However, whether 5-HT or BW723C86 actually stimulates p70S6K activities and the order of activation downstream of TGF-α receptor in primary cultures of adult rat hepatocytes remains unknown.

In the present study, we found that the time course of EGF/TGF-α RTK phosphorylation induced by 5-HT or BW723C86 peaked at about 10 min (Fig. 1), whereas that of p70S6K significantly increased at about 30 min (Fig. 2). These results indicate that p70S6K is a downstream signal-transducing element of the EGF/TGF-α RTK Gq/PLC/Ca^{2+} signaling
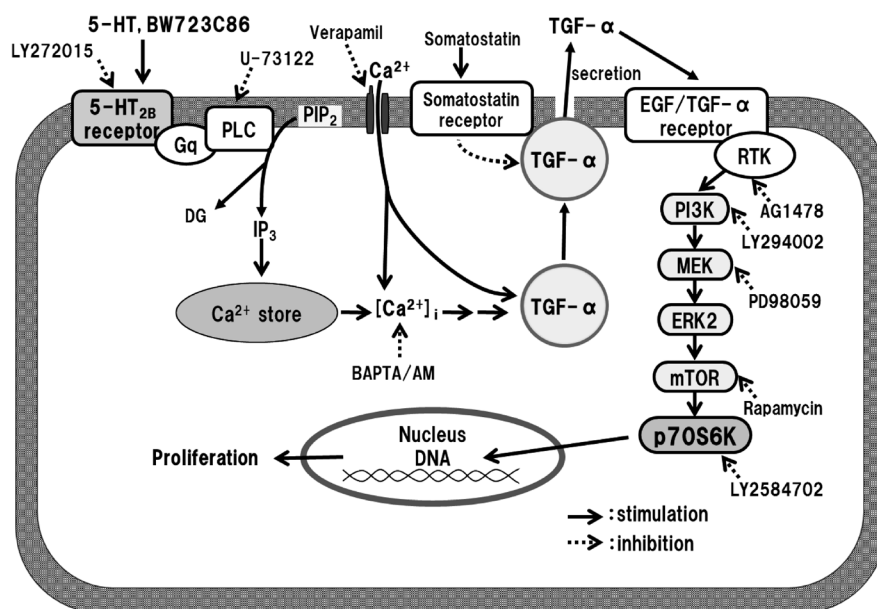
Fig. 6.   Mechanism of 5-HT-Induced DNA Synthesis and Proliferation Was Mediated by the 5-HT$_{2B}$ Receptor/Gq/PLC/Ca^{2+} and EGF/TGF-α RTK/ERK2/p70S6K Pathways in Hepatocytes

   PLC: phospholipase C; PIP$_2$: phosphatidylinositol 4,5-bisphosphate; DG: diacylglycerol; IP$_3$: inositol 1,4,5-triphosphate; RTK: receptor tyrosine kinase; PI3K: phosphoinositide 3-kinase; ERK2: extracellular signal-regulated kinase 2; mTOR: mammalian target of rapamycin; p70S6K: ribosomal p70 S6 kinase; TGF-α: transforming growth factor-α.

pathway.

   In another time course study, a significant increase in TGF-α secretion was seen 3 min after 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) treatment as compared with control. Maximal TGF-α levels were reached at 10 min after the addition of these agents.[14] Moreover, in a previous time course study, we found that both 5-HT and BW723C86 induced an increase in ERK2 but not ERK1 phosphorylation, with the phosphorylated ERK2 band (p-ERK2) peaking at between 20 and 30 min after the addition of these agents.[13]

   In summary, the signal transduction cascade proceeds, at least, in the following order: 5-HT$_{2B}$ receptor stimulation, phosphorylation of p175 RTK (onset; 5–10 min), phosphorylation of ERK2 (onset; 20–30 min), and phosphorylation of p70S6K (onset; 30 min). By contrast, the stimulation of hepatocytes with exogenous EGF (a direct mitogen) induces a rapid increase in EGF/TGF-α RTK phosphorylation, peaking at 3 min after addition.[36] The difference in the time course between 5-HT and EGF is the result of autocrine secretion of TGF-α.

   As shown in Fig. 3, phosphorylation of p70S6K induced by 5-HT or BW723C86 completely blocked the 5-HT$_{2B}$ receptor antagonist LY272015 (10^{-6} M),[22] but not the 5-HT$_{2A}$ receptor antagonist ketanserin (10^{-6} M)[23] or the 5-HT$_{2C}$ receptor antagonist SB242084 (10^{-6} M).[24] These results suggest 5-HT$_{2B}$ receptor-mediation of p70S6K phosphorylation. Moreover, 5-HT- or BW723C86-induced phosphorylation of p70S6K was completely abolished by AG1478 (10^{-6} M),[25] LY294002 (3×10^{-7} M),[26] PD98059 (10^{-6} M),[27] rapamycin (10 ng/mL),[28] and the p70S6K inhibitor LY2584702 (10^{-8} M).[29] As expected, mitogenesis in cultured hepatocytes induced by 5-HT or BW723C86 was blocked by a specific p70S6K inhibitor LY2584702 in a dose-dependent manner after culturing for 4 h (Fig. 4). Rapamycin did not affect phosphorylation of ERK2 induced by 5-HT or BW723C86,[13] which indicated ERK2 is

upstream signal-transducing element of p70S6K. Additionally, phosphorylation of ERK2 or p70S6K were blocked LY294002 but not phosphorylation of p175 RTK. These findings suggest that PI3K mediates the signal from p175 RTK to ERK2 and p70S6K. Phosphorylation of p70S6K induced by 5-HT or BW723C86 was also abolished by U-73122, BAPTA/AM, verapamil, and somatostatin (Fig. 5). These findings indicate that 5-HT- or BW723C86-induced phosphorylation of p70S6K is mediated through the activation of signaling pathways involving 5-HT$_{2B}$ receptor/Gq/PLC/Ca^{2+} and EGF/TGF-α RTK/PI3K/MEK/mTOR; p70S6K is a downstream element of all these signal transducers.

   mTOR signaling is emerging as a major consideration in immunosuppression and cancer chemotherapy, and further evidence of the necessity of the MAPK/mTOR/p70S6K pathway in breast cancer cells and neoplastic hepatic cells has been obtained.[37,38] In addition, proliferation induced by bioactive compounds (*e.g.*, prostaglandins, tumor necrotizing factor-α, and interleukin-1β) is closely associated with the ERK2/mTOR/p706SK pathway in primary cultures of rat adult hepatocytes.[19,39,40] Therefore, p70S6K is an important element of signal transducers that mediates protein synthesis, survival, apoptosis, and advanced hepatocyte mitogenesis.[41–43]

   In conclusion, the findings from the present study suggest that hepatocyte proliferation in culture is stimulated by 5-HT through the activation of the 5-HT$_{2B}$/Gq/PLC/Ca^{2+} and EGF/TGF-α RTK/ERK2/mTOR/p70S6K signaling pathways (Fig. 6). Because 5-HT is known to be released by activated platelets as a result of liver injury or damage, liver regeneration may be enhanced by platelet-derived 5-HT through 5-HT$_{2B}$ receptor stimulation *in vivo*.

Conflict of Interest   The authors declare no conflict of interest.

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