Regular Article

Signal Transduction Mechanism for Serotonin 5-HT_{2B} Receptor-Mediated DNA Synthesis and Proliferation in Primary Cultures of Adult Rat Hepatocytes

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The involvement of serotonin (5-hydroxytryptamine; 5-HT) and the 5-HT_2 receptor subtypes in the induction of DNA synthesis and proliferation was investigated in primary cultures of adult rat hepatocytes to elucidate the intracellular signal transduction mechanisms. Hepatocyte parenchymal cells maintained in a serum-free, defined medium, synthesized DNA and proliferated in the presence of 5-HT or a selective 5-HT_{2B} receptor agonist, BW723C86, but not in the presence of 5-HT_{2A}, or 5-HT_{2C} receptor agonists (TCB-2 and CP809101, respectively), in a time- and dose-dependent manner. A selective 5-HT_{2B} receptor antagonist, LY272015 (10^{-7} M), and a specific phospholipase C (PLC) inhibitor, U-73122 (10^{-6} M), as well as specific inhibitors of growth-related signal transducers—including AG1478, LY294002, PD98059, and rapamycin—completely inhibited 5-HT (10^{-6} M)- or BW723C86 (10^{-6} M)-induced hepatocyte DNA synthesis and proliferation. Both 5-HT and BW723C86 were shown to significantly stimulate the phosphorylation of epidermal growth factor (EGF)/transforming growth factor (TGF)-α receptor tyrosine kinase (p175 kDa) and extracellular signal-regulated kinase (ERK) 2 on Western blot analysis. These results suggest that the proliferative mechanism of activating 5-HT is mediated mainly through 5-HT_{2B} receptor-stimulated Gq/PLC and EGF/TGF-α-receptor/phosphatidylinositol 3-kinase (PI3K)/ERK2/mammalian target of rapamycin (mTOR) signaling pathways in primary cultured hepatocytes.

Key words serotonin (5-hydroxytryptamine); signal transduction; DNA synthesis; proliferation (cultured hepatocyte); transforming growth factor-α

Although adult rat hepatocytes rarely undergo cell division under normal physiological conditions, due to the ensuing regenerative response to recruit mature hepatocytes into the cell cycle *in vivo* after 70% partial hepatectomy, a phenomenon known as liver regeneration,[1,2] they do remain capable of proliferation. Among the numerous growth-promoting factors that pertain to liver regeneration, platelet-derived serotonin (5-hydroxytryptamine; 5-HT) is reportedly directly involved.[3–7]

5-HT is not only a neurotransmitter, but also a kind of hormone with various extraneural functions. 5-HT mediates a diverse array of responses—including platelet aggregation, vascular constriction, and cell proliferation—by interacting with 5-HT receptor subtypes in mammals.[8] Seven receptor classes including 14 subtypes of 5-HT receptors have been identified to date, reflecting the diversity of 5-HT actions. With the exception of the 5-HT_3 receptors, which are ligand-gated ion channels, all known 5-HT receptors are G-protein-coupled receptors that are positively linked to phosphatidylinositol turnover or cyclic AMP production and are further linked to a variety of downstream pathways.[9–11]

In the presence of epidermal growth factor (EGF) and insulin, 5-HT has been shown to cause a dose-dependent increase in DNA synthesis in primary cultures of rat hepatocytes, suggesting that it is also a co-mitogen.[12] In addition, interaction has also been seen between 5-HT and various hematological factors such as hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF). Although 5-HT has been shown to play significant roles in the stimulation and acceleration of hepatocyte proliferation,[6] few studies have been conducted on the possible involvement of 5-HT_2 receptor subtypes and their intracellular signal transduction pathways in 5-HT-induced hepatocyte DNA synthesis and proliferation in primary cultures of adult rat hepatocytes.

Some recently identified selective 5-HT receptor agonists and antagonists have shown promise as useful tools for differentiating between closely related 5-HT_2 receptor subtypes.[8,13–16] Therefore, in this study, we used these agents to investigate the involvement of 5-HT_2 receptor subtypes and their intracellular signal transduction pathways in 5-HT-induced hepatocyte DNA synthesis and proliferation in primary cultures of adult rat hepatocytes.

MATERIALS AND METHODS

Animals Male Wistar rats (weight range, 200–220 g) were purchased from Tokyo Experimental Animal Co. (Tokyo, Japan) and adapted to a light-, humidity- and temperature-controlled room over a minimum 3-d period prior to the start of the experiments. All rats were fed with a standard laboratory diet and given tap water *ad libitum*. All rats used in this study were given human care in compliance with Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society and Josai University.

Hepatocyte Isolation and Culture Rats were anesthetized intraperitoneally with sodium pentobarbital (45 mg/kg). To facilitate disaggregation of the adult rat liver, normal

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hepatocytes were isolated by the two-step *in situ* collagenase perfusion technique described by Seglen.[17] All hepatocytes displayed a viability of >97% as determined by Trypan blue exclusion.

Freshly isolated hepatocytes were plated onto 35-mm diameter collagen-coated plastic culture dishes (Iwaki Glass Co., Tokyo, Japan) at a density of 3.3×10^4 cells/cm^2, and then cultured for 3 h in Williams' medium E containing 5% newborn calf serum, 0.1 nM dexamethasone, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.10 μg/mL aprotinin in 5% CO_2 in air at 37°C, as described previously.[18] Next, the medium was removed by aspiration and the cells were cultured in serum- and dexamethasone-free Williams' medium E supplemented with 5-HT or selective 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptor agonists (*i.e.*, TCB-2, BW723C86, and CP809101, respectively). The following agents were then added as appropriate: 5-HT or selective 5-HT receptor subtype agonists with or without selective 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptor antagonists (ketanserin, LY272015, and SB242084, respectively), U-73122, U-73343, GF109203X, H-89, 2,4-dideoxyadenosine (DDA), BAPTA/AM, verapamil, somatostatin, and growth-related signal transducer inhibitors (AG1478, LY294002, PD98059, and rapamycin).

Measurement of DNA Synthesis and Cell Proliferation To assess hepatocyte DNA synthesis, the incorporation of [^{3}H]thymidine into acid-precipitable materials was examined,[19] as previously described.[18] Briefly, after an initial attachment period of 3 h, parenchymal hepatocytes were washed twice with serum-free Williams' medium E and cultured for an additional 4 h in medium containing 5-HT or selective 5-HT receptor subtype agonists. Two hours after the addition of the agonists, cells were pulse-stimulated with [^{3}H]-thymidine (1.0 μCi/well) for another 2 h. Data are expressed as dpm/h·mg cellular protein, which was determined using a modified Lowry procedure with bovine serum albumin as a standard.[20] The number of nuclei rather than the number of cells was counted, as described previously.[18]

Determination of Receptor Tyrosine Kinase Activity Immunoblotting with corresponding anti-phospho-receptor tyrosine kinase antibody according to the manufacturer's instructions, as previously described,[21] identified a 175-kDa protein as the EGF/TGF-α receptor. Regarding immunoblotting analysis, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in a 7.5% polyacrylamide resolving gel[22] was used to analyze samples of the supernatant (30 μg/lane), which were then transferred to polyvinylidene difluoride (PVDF) membrane and immunoblotted with PY 20, an anti-phosphotyrosine antibody.[23] Blots were developed using enhanced chemiluminescence reagents (PerkinElmer, Inc., MA, U.S.A.) after incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies, as previously described.[21] After the membrane was developed with enhanced chemiluminescence reagent and exposure to Hyperfilm (Kodak, Tokyo), proteins were quantified using densitometry. The NIH image program (ver. 1.6 for Macintosh) was used for densitometric analysis. Next, the phosphorylated p175-kDa protein (P-p175 kDa) tyrosine kinase activity was normalized to that of the total p175-kDa protein. A modified Lowry procedure with bovine serum albumin as a standard was used to determine the supernatant protein concentration.[20]

Determination of Extracellular Signal-Regulated Kinase (ERK1/2) Activity Excluding the application of 20 μg of the supernatant per lane of a 10% polyacrylamide resolving gel, cell lysis and Western blotting procedures were carried out as described in the previous section. Western blotting analysis with an anti-phospho-ERK1/2 monoclonal antibody[24] was used to identify phosphorylated ERK isoforms (pERK1; P-p44 mitogen-activated protein kinase (MAPK) and pERK2; P-p42 MAPK), and then phosphorylated ERK (p-ERK) activity was normalized to the total ERK activity. Data were calculated in arbitrary units and are expressed as the mean±standard error of the mean (S.E.M.). The autodiagram is a representation of three experiments using different cell preparations. Cytosolic protein in hepatocytes was quantified as described above.[20]

Materials Serotonin hydrochloride (5-HT), aphidicolin, dexamethasone, somatostatin, verapamil hydrochloride, and aprotinin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Selective agonists for 5-HT_2 receptor subtypes were obtained as follows: TCB-2 (Tocris Bioscience, Bristol, U.K.); BW723C86 (Sigma Chemical Co.); and CP809101 hydrochloride (Tocris Bioscience). Selective antagonists for 5-HT_2 receptor subtypes were obtained as follows: ketanserin tartrate (Enzo Life Sciences, Farmingdale, NY, U.S.A.); LY272015 hydrochloride (Santa Cruz Biotechnology, Dallas, TX, U.S.A.); and SB242084 (Tocris Bioscience). BAPTA/AM [1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester)] was obtained from Santa Cruz Biotechnology. U-73122 (1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino]hexyl]-1*H*-pyrrol-2,5-dione), U-73343 (1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidine-dione), GF109203X (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide), 2,4-dideoxyadenosine, H-89 (*N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride), AG1478 (2-[4-morpholinyl]-8-phenyl-1(4*H*)-benzopyran-4-one), LY294002 (N-[3-chlorophenyl]-6,7-dimethoxy-4-quinazolinamine), and rapamycin were obtained from Enzo Life Sciences. PD98059 (2′-amino-3′-methoxyflavone) was obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.). Williams' medium E and newborn calf serum were purchased from Flow Laboratories (Irvine, Scotland). Collagenase (type II) was obtained from Worthington Biochemical Co. (Freehold, NJ, U.S.A.). [Methyl-^{3}H] thymidine (20 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA, U.S.A.). All other reagents were of analytical grade.

Data Analysis and Statistics Data are expressed as 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. p values <0.05 were considered to indicate statistically significance.

RESULTS

Time Course of 5-HT- or BW723C86-Induced Stimulation of Hepatocyte DNA Synthesis and Proliferation The effects of 5-HT on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes were investigated and compared with those of BW723C86, a selective 5-HT_{2B} receptor agonist.[25] Three hours after plating, when the change to serum-free culture medium was made, 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) was added (Fig. 1). Hepatic parenchymal cells underwent time-dependent DNA synthesis and prolifera-

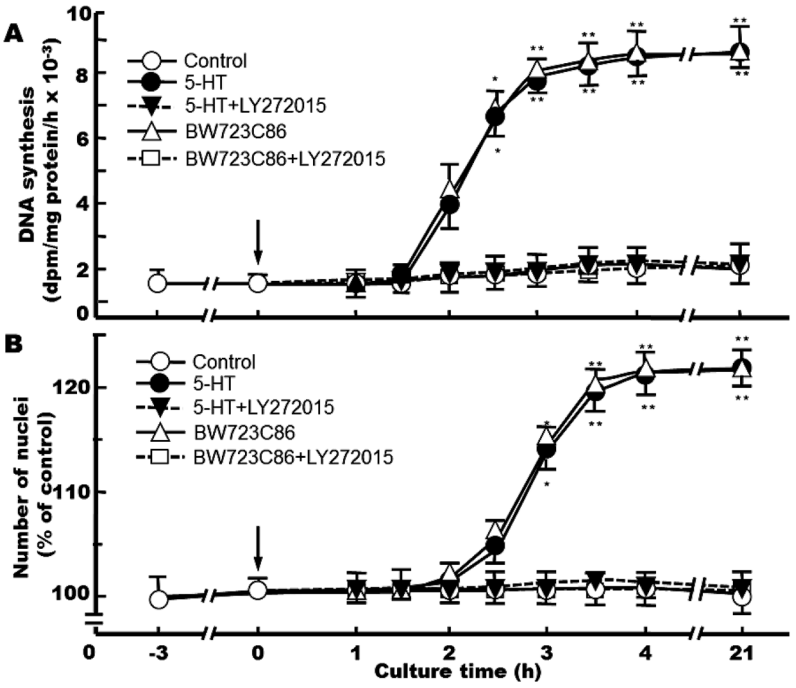

Fig. 1. Time Course of 5-HT- or BW723C86-Induced Hepatocyte DNA Synthesis and Proliferation

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 for various times with 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) with or without LY272015 (10^{-7} M). Hepatocyte DNA synthesis (A) and proliferation (B) were determined as described in Materials and Methods. Results are expressed as mean±S.E.M. of three separate experiments. *$p<0.05$, **$p<0.01$ compared with control.

tion in the presence of 5-HT or BW723C86 when maintained for a short time in the culture medium. Onset of DNA synthesis was observed at about 2.5 h after the addition of 5-HT or BW723C86, and peaked at about 3.5 h (Fig. 1A). Meanwhile, significant proliferative activity was observed at 3 h, peaking at about 4 h (Fig. 1B). The maximum stimulation of hepatocyte DNA synthesis and proliferation induced by 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) was about 6.0- and 1.2-fold, respectively. LY272015 (10^{-7} M), a selective 5-HT$_{2B}$ receptor antagonist, almost completely inhibited the DNA synthesis and proliferative effects of 5-HT (10^{-6} M) and BW723C86 (10^{-6} M) on primary cultured hepatocytes. However, LY272015 (10^{-7} M) alone had no significant effects on DNA synthesis and proliferation in primary cultured hepatocytes (data not shown).

Dose–Response Effects of 5-HT or Selective 5-HT$_2$ Receptor Agonists on Hepatocyte DNA Synthesis and Proliferation The dose–response effects of 5-HT on hepatocyte mitogenesis were then examined and compared with those of BW723C86. 5-HT or BW723C86-induced [^{3}H]thymidine incorporation (*i.e.*, DNA synthetic activity) was found to be dose-dependent, reaching a plateau at a concentration of 10^{-6} M, with half-maximal effective concentration (ED_{50}) values of 1.85×10^{-7} M and 6.89×10^{-8} M for 5-HT and BW723C86, respectively (Figs. 2A, B). The proliferative effects of 5-HT or BW723C86 on cultured hepatocytes were comparable to those on DNA synthesis (Figs. 2A, B). In contrast, hepatocyte DNA synthesis and proliferation were not significantly affected by TCB-2[26], a selective 5-HT$_{2A}$ receptor agonist, or CP809101,[27] a 5-HT$_{2C}$ receptor agonist, within the concentration range of 10^{-10} to 10^{-5} M (Figs. 2A, B).

Effects of Selective Antagonists of 5-HT$_2$ Receptor Subtypes or Specific Inhibitors of Growth-Related Signal Transducers on Hepatocyte DNA Synthesis and Proliferation Induced by 5-HT or BW723C86 Next, the effects of selective 5-HT$_2$ receptor subtype antagonists and specific inhibitors of growth-related signal transducers on hepatocyte DNA synthesis and proliferation induced by 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) were examined. As shown in Fig. 1, the addition of LY272015 (5-HT$_{2B}$ receptor antagonist, 10^{-7} M) almost completely blocked 5-HT- or BW723C86-induced hepatocyte mitogenesis. In contrast, the addition of submaximal concentrations of ketanserin (5-HT$_{2A}$ receptor antagonist, 10^{-6} M) or SB242084 (5-HT$_{2C}$ receptor antagonist, 10^{-6} M) did not affect 5-HT- or BW723C86-induced hepatocyte DNA synthesis and proliferation.

Next, we used AG1487 (a specific inhibitor of EGF/TGF-α receptor tyrosine kinase, 10^{-6} M),[28] LY294002 (a specific inhibitor of phosphatidylinositol 3-kinase (PI3K), 3×10^{-7} M),[29] PD98059 (a specific inhibitor of mitogen-activated protein extracellular kinase (MEK) that is upstream of ERK, 10^{-6} M),[30,31] and rapamycin (a specific inhibitor of mammalian target of rapamycin (mTOR), 10 ng/mL),[32] to investigate whether signal transducers—such as receptor tyrosine kinases (RTKs), PI3K, extracellular signal-regulated kinase (ERK), and mTOR, an upstream element of ribosomal protein p70 S6 kinase—mediated the mitogenic responses of hepatocytes to 5-HT (10^{-6} M) or BW723C86 (10^{-6} M). AG1487 (10^{-6} M), LY294002 (3×10^{-7} M), PD98059 (10^{-6} M), and rapamycin (10 ng/mL) almost completely inhibited the 5-HT- or BW723C86-induced hepatocyte DNA synthesis and proliferation during the early phase (4 h) of culture (Figs. 3A, B), suggesting the involvement of receptor tyrosine kinase, PI3K, ERK, and mTOR in these processes. Next, since PLC is reportedly involved in 5-HT$_2$ receptor subtype signaling, the PLC inhibitor U-73122[33] was used to investigate the involvement of phospholipase C (PLC) in 5-HT (10^{-6} M)- or BW723C86 (10^{-6} M)-induced hepatocyte

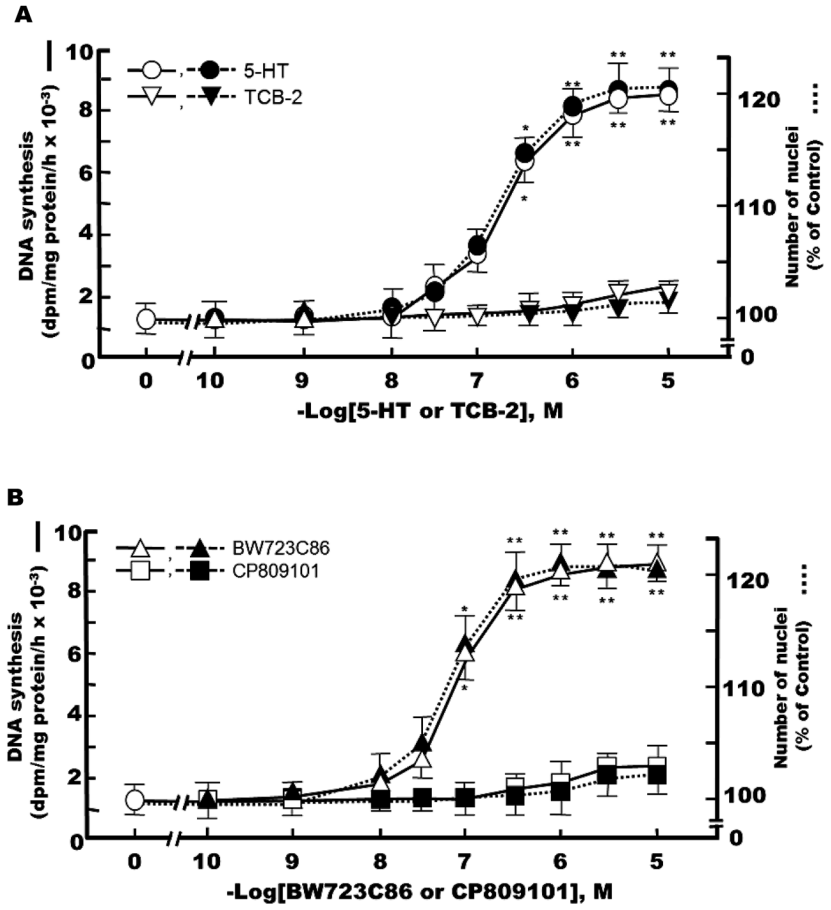

Fig. 2. Dose–Response Effects of 5-HT or Selective 5-HT_2 Receptor Agonists on Hepatocyte DNA Synthesis and Proliferation

Hepatocytes were plated at a cell density of 3.3×10^4 cells/cm^2 and cultured for 3 h as described in the legend to Fig. 1. After replacing the medium, the cultured hepatocytes were treated for 4 h with increasing concentrations of 5-HT (10^{-10} M to 10^{-5} M) or TCB-2 (10^{-10} M to 10^{-5} M) (A), and BW723C86 (10^{-10} M to 10^{-5} M) or CP809101 (10^{-10} M to 10^{-5} M) (B). DNA synthesis (opened symbols) and cell proliferation (closed symbols) were assessed as described in Materials and Methods. Results are expressed as mean±S.E.M. of three separate experiments. *$p<0.05$, **$p<0.01$ compared with control.

DNA synthesis and proliferation. Whereas U-73122 (10^{-6} M) attenuated 5-HT- and BW723C86-induced hepatocyte DNA synthesis and proliferation (Fig. 3), the inactive structural analogue U-73343 (10^{-6} M) did not (data not shown). In addition, both somatostatin (10^{-7} M) and BAPTA/AM (10^{-7} M),[34] the membrane-permeable chelator of Ca^{2+}, inhibited 5-HT- and BW723C86-induced hepatocyte DNA synthesis and proliferation; this suggested that both protein secretion and calcium signaling play a role in these processes. Hepatocyte DNA synthesis and proliferation was not affected by any of the specific inhibitors independently (data not shown).

Effects of Selective Antagonists of 5-HT_2 Receptor Subtypes or Specific Inhibitors of Growth-Related Signal Transducers on the Phosphorylation of the EGF/TGF-α Receptor Tyrosine Kinase (p-175 kDa) Induced by 5-HT or BW723C86 Next, in order to confirm whether hepatocyte DNA synthesis and proliferation is induced by 5-HT or BW723C86 through the EGF/TGF-α receptor tyrosine kinase (p-175 kDa)/ERK signaling pathway, we investigated whether 5-HT or BW723C86 could stimulate phosphorylation of the EGF/TGF-α receptor tyrosine kinase. We found that 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) caused an increase in the phosphorylation of the EGF/TGF-α receptor tyrosine kinase (p175 kDa), which peaked at about 2.9-fold the level of control at 10 min after addition (Fig. 4A), and was completely antagonized by LY272015 (10^{-7} M). In addition, no effect was observed with the addition of ketanserin (10^{-6} M) or SB242084 (10^{-6} M) to 5-HT or BW723C86. However, the 5-HT- or BW723C86-induced increase in phosphorylation was almost completely inhibited with the addition of U-73122 (10^{-6} M), a PLC inhibitor, suggesting that the 5-HT_{2B} receptor/Gq/PLC pathway is closely associated with the induced putative mitogen secretion by cultured hepatocytes. U-73343 (10^{-6} M), an inactive structural analogue of U-73122, was found to have no effect on the 5-HT- or BW723C86-induced increases in phosphorylation of the EGF/TGF-α receptor tyrosine kinase.

The addition of AG1478 (10^{-6} M) with 5-HT or BW723C86 significantly inhibited the 5-HT- or BW723C86-induced phosphorylation of the EGF/TGF-α receptor tyrosine kinase. In contrast, the addition of a growth-inhibiting dose of LY294002, PD98059, or rapamycin with 5-HT or BW723C86 had no effect on the induced phosphorylation of the EGF/TGF-α receptor tyrosine kinase (Fig. 4A). Therefore, the phosphorylation of the EGF/TGF-α receptor tyrosine kinase was not independently affected by these inhibitors (data not shown). 5-HT_{2B} receptor mediated hepatocyte mitogenesis upstream of RTK phosphorylation.

Hepatocytes were then treated with verapamil (L-type Ca^{2+} channel blocker, 10^{-6} M), somatostatin (10^{-7} M), and BAPTA/AM (a membrane-permeable Ca^{2+} chelator, 10^{-7} M) for 10 min to determine the degree of involvement of Ca^{2+} mobilization in the 5-HT- or BW723C86-induced phosphorylation of

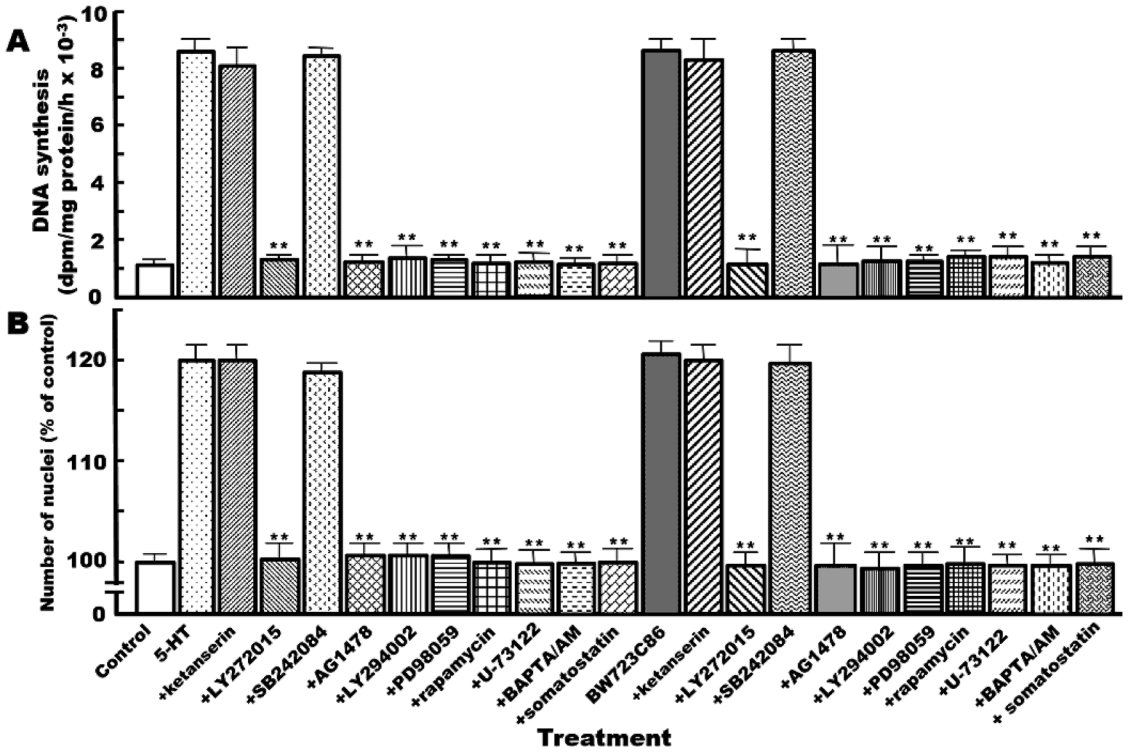

Fig. 3. Effects of Selective Antagonists of 5-HT_2 Receptor Subtypes or Specific Inhibitors of Growth-Related Signal Transducers on 5-HT- or BW723C86-Induced Hepatocyte DNA Synthesis and Proliferation

Hepatocytes were plated at a cell density of 3.3×10^4 cells/cm^2 and cultured for 3 h as described in the legend to Fig. 1. After replacing the medium, the hepatocytes were cultured for an additional 4 h with 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) in the presence of 5-HT_2 receptor subtype antagonists or specific inhibitors of growth-related signal transducers. The concentrations of antagonists/inhibitors used were as follows: ketanserin (10^{-6} M), LY272015 (10^{-7} M), SB242084 (10^{-6} M), U-73122 (10^{-6} M), AG1478 (10^{-6} M), LY294002 (3×10^{-7} M), PD98059 (10^{-6} M), rapamycin (10 ng/mL), BAPTA/AM (10^{-7} M), and somatostatin (10^{-7} M). Hepatocyte DNA synthesis (A) and proliferation (B) were determined as described in Materials and Methods. Data are expressed as mean±S.E.M. of three separate experiments. $^{**}p<0.01$ compared with the respective control.

the EGF/TGF-α receptor tyrosine kinase; significant inhibition was observed following treatment with somatostatin (10^{-7} M) or verapamil (10^{-6} M) (Fig. 4B). Moreover, the 5-HT or BW723C86-induced phosphorylation of the EGF/TGF-α receptor tyrosine kinase was not affected by GF109203X (an inhibitor of PKC),[35] dideoxyadenosine (a direct inhibitor of adenylate cyclase),[36] or H-89 (an inhibitor of PKA).[37] These inhibitors and stimulators did not independently influence phosphorylation of the EGF/TGF-α receptor tyrosine kinase during 1 h of culture (data not shown).

Time–Course and Patterns of 5-HT or BW723C86 Stimulation of ERK Isoform Phosphorylation Next, the time–course and patterns of 5-HT or BW723C86 stimulation of ERK isoform phosphorylation in primary cultured hepatocytes, as detected by Western blotting, were examined. The results of the time–course study suggested that a phosphorylated ERK2 band (pERK2) was significantly induced after 20 min stimulation, peaking (about a 3-fold increase compared with a control) at 30 min after addition of 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) (Figs. 5A, B). Phosphorylation then gradually declined to basal levels over 1 h. ERK1 phosphorylation was not significantly affected by treatment with either medium independently or by 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) (control) (Figs. 5A, C).

Effects of Selective Antagonists of 5-HT_2 Receptor Subtypes or Specific Inhibitors of Growth-Related Signal Transducers on ERK Phosphorylation Induced by 5-HT or BW723C86 Next, we examined the effects of selective antagonists of 5-HT_2 receptor subtypes or specific inhibitors of growth-related signal transducers on ERK2 phosphorylation induced by 5-HT or BW723C86 at 30 min in order to obtain further evidence for ERK mediation of induced hepatocyte mitogenesis. As shown in Fig. 6, 5-HT or BW723C86 caused an increase in the phosphorylation of ERK2, but not ERK1. The 5-HT_{2B} receptor antagonist, LY272015 (10^{-7} M), almost completely inhibited the phosphorylation of ERK2 induced by 5-HT (10^{-6} M) or BW723C86 (10^{-6} M). PD98059 (10^{-6} M) completely abolished the 5-HT- or BW723C86-induced ERK2 phosphorylation when combined with 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) (Fig. 6A). Moreover, ERK2 phosphorylation was abolished by AG1478 (10^{-6} M), the EGF/TGF-α receptor tyrosine kinase inhibitor, or LY294002 (3×10^{-7} M), the PI3K inhibitor, but not by rapamycin (10 ng/mL) (Fig. 6A). These results suggested that the mitogenic effects of 5-HT or BW723C86 were mediated through the activation of a receptor tyrosine kinase/PI3K/ERK2 pathway. U-73122, but not U-73343, completely blocked the 5-HT- or BW723C86-induced phosphorylation of ERK2. Verapamil (10^{-6} M), BAPTA/AM (10^{-7} M), and somatostatin (10^{-7} M) (Fig. 6B) also completely blocked the 5-HT- or BW723C86-induced phosphorylation of ERK2, which suggests that the 5-HT_{2B} receptor/(Gq)/PLC/Ca^{2+} pathway also stimulates the secretion of a putative mitogen by primary cultured hepatocytes in an autocrine manner. On the other hand, 5-HT- or BW723C86-induced ERK2 phosphorylation was not affected by GF109203X (10^{-6} M), DDA (10^{-6} M), or H-89 (10^{-6} M) (Fig. 6B).

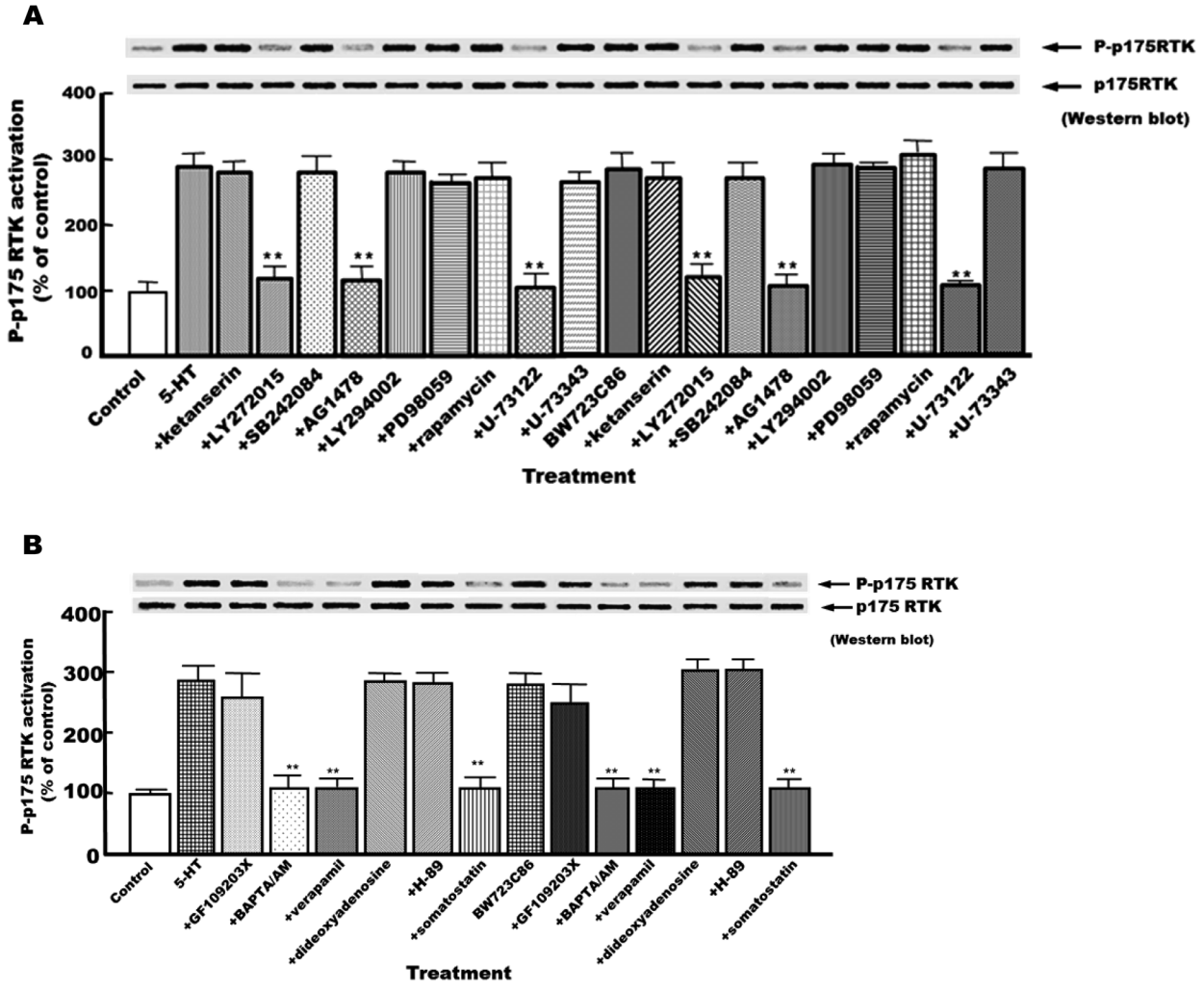

Fig. 4. Effects of Selective Antagonists of 5-HT_2 Receptor Subtypes or Specific Inhibitors of Growth-Related Signal Transducers on 5-HT- or BW723C86-Induced Phosphorylation of EGF/TGF-α Receptor Tyrosine Kinase (p-175 kDa)

Hepatocytes were plated at a cell density of 3.3×10^4 cells/cm^2 and cultured for 3 h as described in the legend to Fig. 1. Next, the medium was rapidly replaced with serum-free Williams' medium E, and the hepatocytes were cultured for various times with 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) with or without inhibitors of growth-related signal transducers for 10 min. 5-HT- or BW723C86-induced phosphorylation of EGF/TGF-α receptor tyrosine kinase (p175 kDa) is described in Materials and Methods. Western blot analysis is shown at the top of each panel. Concentrations of agents used were as follows: (A) 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), U-73122 (10^{-6} M), and U-73343 (10^{-6} M); (B) GF109203X (10^{-6} M), BAPTA/AM (10^{-7} M), verapamil (10^{-6} M), dideoxyadenosine (10^{-6} M), H-89 (10^{-6} M), and somatostatin (10^{-7} 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 the respective control.

DISCUSSION

Based on our results using 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptor subtype-specific agonists and antagonists, the 5-HT_{2B} receptor, but not the 5-$HT_{2A,}$ or 5-HT_{2C} receptors, mediate a 5-HT time- and dose-dependent increase in DNA synthesis and proliferation in primary cultures of adult rat hepatocytes (Figs. 1–3). These results clearly indicate that 5-HT is a primary mitogen, but not a co-mitogen, as reported by Balasubramanian and Paulose.[12] Moreover, our findings that the 5-HT_{2B} receptor subtype is involved in the promotion of liver regeneration *in vivo* are consistent with those of Lesurtel *et al.*[4]

We also investigated whether post-receptor mechanisms might have been responsible for the 5-HT_{2B} receptor subtype-mediated proliferative action. We found that specific inhibitors of growth-related signal transducers—including U-73122 (a specific inhibitor of PLC), AG1478 (a specific inhibitor of EGF/TGF-α receptor tyrosine kinase), LY294002 (a specific inhibitor of PI3K), PD98059 (a specific inhibitor of ERK), and rapamycin (a specific inhibitor of mTOR)—attenuated 5-HT- or BW723C86-induced hepatocyte DNA synthesis and proliferation (Fig. 3). These findings suggest that, at a minimum, mitogenic signaling through a 5-HT_{2B} receptor pathway involves activation of Gq/PLC, EGF/TGF-α-RTK, PI3K, ERK, and mTOR, as well as putative protein secretion and calcium signaling (Fig. 3).

In addition, 5-HT_{2B} receptors have been reported to be G-protein-linked receptors positively coupled to phosphatidylinositol metabolism.[11] Therefore, the association between the 5-HT_{2B} receptor/(Gq)/PLC pathway and the RTK/PI3K/ERK/mTOR pathway in 5-HT_{2B} receptor-mediated hepatocyte mitogenesis remains unclear.

Prostaglandin EP_1 and IP receptor agonists were shown to induce hepatocyte DNA synthesis and proliferation through the induction of autocrine secretion of the primary mitogen TGF-α in previous studies.[38,39] This in turn stimulates hepatocyte mitogenesis through a EGF/TGF-α receptor tyrosine

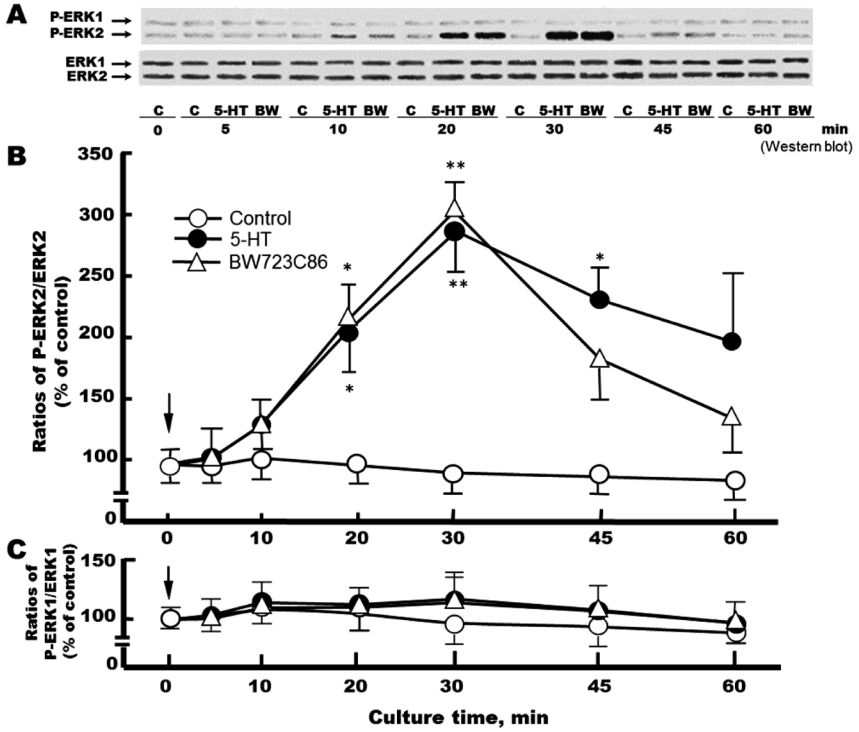

Fig. 5. Time–Course of 5-HT or BW723C86 Stimulation of ERK Isoform Phosphorylation

Hepatocytes were plated at a cell density of 3.3×10^4 cells/cm^2 and cultured for 3 h as described in the legend to Fig. 1. 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. Phosphorylation of ERK 1/2 by 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) is described in Materials and Methods. (A) Western blot image, (B) ERK2 phosphorylation, and (C) ERK1 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.

kinase/ERK pathway. In this study, we hypothesized that the secretion of a putative complete mitogen by primary cultured hepatocytes was also stimulated by the 5-HT$_{2B}$ receptor/(Gq)/PLC/Ca^{2+} pathway in an autocrine manner, leading to the induction of hepatocyte DNA synthesis and cell proliferation through the stimulation of a downstream receptor tyrosine kinase (RTK)/ERK pathway. Because hepatocytes express mRNA for both TGF-α and insulin-like growth factor (IGF)-I, and the cells are capable of synthesizing and storing these primary mitogens,[1] TGF-α and IGF-I are considered to be reasonable candidates for primary mitogens.

Furthermore, 5-HT- or BW723C86-induced hepatocyte mitogenesis was completely inhibited by the PLC inhibitor, U-73122,[32] but not by its inactive analog, U-73343 (Fig. 3). Therefore, PLC and the subsequent increase in Ca^{2+} mobilization from stored sites are likely stimulated by the functional 5-HT$_{2B}$ receptors found in hepatocytes in primary cultures to secrete a putative mitogen as a primary mechanism of 5-HT$_{2B}$ receptor agonist-induced hepatocyte mitogenesis (Fig. 3). In addition, verapamil, the L-type Ca^{2+} channel blocker (data not shown), BAPTA/AM, the cell-permeable Ca^{2+} chelator, and somatostatin, which inhibits the release of certain pancreatic hormones, were found to inhibit hepatocyte DNA synthesis and proliferation induced by 5-HT or BW723C86, providing further evidence for the involvement of intracellular Ca^{2+} (Fig. 3). Therefore, these growth-related signal transducer inhibitors (AG1478, LY294002, PD98059, and rapamycin) appear to block mitogenic 5-HT$_{2B}$ receptor signaling by inhibiting corresponding growth-related signal transducer (RTK, PI3K, ERK, and mTOR) activities induced by a putative mitogen, which in turns blocks hepatocyte DNA synthesis and proliferation (Figs. 3A, B). These findings suggest that the promotion of hepatocyte mitogenesis by 5-HT or BW723C86 is due to a secondary effect of the secreted putative mitogen that occurred in an autocrine manner *via* stimulation of the 5-HT$_{2B}$ receptor/Gq protein/PLC pathway.

We then sought to confirm whether 5-HT or BW723C86 induces hepatocyte DNA synthesis and proliferation through an EGF/TGF-α receptor tyrosine kinase/ERK signaling pathway. First, we showed that the addition of 5-HT or BW723C86 led to an increase in the phosphorylation of the EGF/TGF-α receptor tyrosine kinase (p175 kDa), peaking at about 2.9-fold (compared with control) at 10 min (Fig. 4). We then showed that 5-HT- or BW723C86-induced phosphorylation of the EGF/TGF-α receptor tyrosine kinase was completely antagonized by LY272015 (10^{-7} M), a selective 5-HT$_{2B}$ receptor antagonist, and by the RTK and PLC inhibitors, AG1478 (10^{-6} M) and U-73122, respectively. LY272015 blocked the phosphorylation of a 175 kDs receptor tyrosine kinase substrate protein, indicating that the 5-HT$_{2B}$ receptor activates RTK phosphorylation *via* a putative mitogen. In contrast, 5-HT- or BW723C86-induced phosphorylation of the EGF/TGF-α receptor tyrosine kinase was not affected by LY294002, PD98059, or rapamycin, suggesting that PI3K, ERK, and mTOR are downstream elements of RTK signaling.

Further evidence for the ERK2 mediation of 5-HT activity was obtained in additional experiments (Figs. 5, 6). In these experiments, 5-HT (10^{-6} M) or BW723C86 (10^{-6} M) were shown to induce the phosphorylation of ERK2, which was specifically inhibited by PD98059 (10^{-6} M) and completely inhibited by LY272015. U-73122 (10^{-6} M), verapamil (10^{-6} M), and somatostatin (10^{-7} M) inhibited 5-HT- or BW723C86-induced RTK

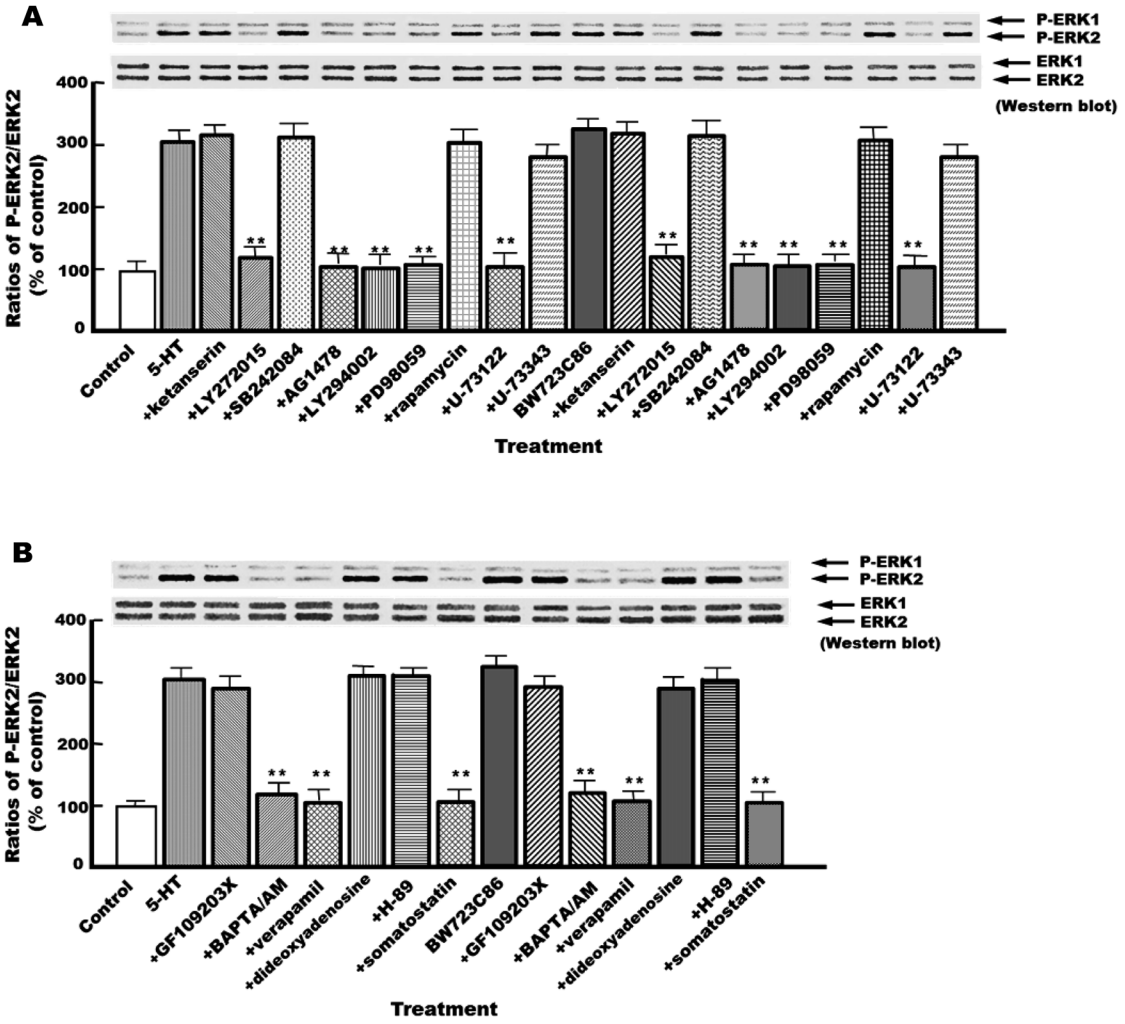

Fig. 6. Effects of Selective Antagonists of 5-HT_2 Receptor Subtypes or Specific Inhibitors of Growth-Related Signal Transducers on 5-HT- or BW723C86-Induced ERK Phosphorylation

Hepatocytes were plated at a cell density of 3.3×10^4 cells/cm^2 and cultured for 3 h as described in the legend to Fig. 1. 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 inhibitors of growth-related signal transducers for 30 min. Phosphorylation of ERK 1/2 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: (A), 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), and rapamycin (10 ng/mL), U-73122 (10^{-6} M), and U-73343 (10^{-6} M); (B), GF109203X (10^{-6} M), BAPTA/AM (10^{-7} M), verapamil (10^{-6} M), dideoxyadenosine (10^{-6} M), H-89 (10^{-6} M), and somatostatin (10^{-7} 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 respective control.

phosphorylation and ERK2 phosphorylation; therefore, the 5-HT or BW723C86-stimulated responses were mediated by PLC and extracellular Ca^{2+}, which is closely associated with cell proliferation in primary cultures of adult rat hepatocytes (Fig. 6B). In contrast, GF109203X, DDA, and H-89 did not affect 5-HT-induced RTK and ERK2 phosphorylation; therefore, protein kinase C and adenylate cyclase/protein kinase A were not involved in these processes (Fig. 6B). We also found that 5-HT or BW723C86 did not significantly stimulate ERK1 phosphorylation (Figs. 5, 6).

In this study involving primary cultures of adult rat hepatocytes, we demonstrated that 5-HT is a primary mitogen, but not a co-mitogen. Moreover, in relation to post-receptor mechanisms, the 5-HT_{2B} receptor/(Gq)/PLC/Ca^{2+}-pathway-dependent autocrine secretion of a putative mitogen was shown to be essential in the induction of DNA synthesis and proliferation in primary cultured hepatocytes. Additional studies on the secretion of a putative complete mitogen in an autocrine manner should be conducted in the future.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Michalopoulos GK, DeFrances MC. Liver regeneration. *Science*, **276**, 60–66 (1997).
2) Fausto N, Campbell JS, Riehle KJ. Liver regeneration. *Hepatology*, **43** (Suppl. 1), S45–S53 (2006).
3) Jeyabalan G, Geller DA. The importance of platelet-derived serotonin in mediating hepatic regeneration. *J. Hepatol.*, **45**, 629–630 (2006).
4) Lesurtel M, Graf R, Aleil B, Walther DJ, Tian Y, Jochum W, Gachet C, Bader M, Clavien PA. Platelet-derived serotonin mediates liver regeneration. *Science*, **312**, 104–107 (2006).
5) Lesurtel M, Clavien P-A. Serotonin: a key molecule in acute and chronic liver injury. *Clin. Res. Hepatol. Gastroenterol.*, **36**, 319–322 (2012).
6) Murata S, Ohkohchi N, Matsuo R, Ikeda O, Myronovych A, Hoshi R. Platelets promote liver regeneration in early period after hepatec-

tomy in mice. *World J. Surg.*, **31**, 808–816 (2007).
7) Papadimas GK, Tzirogiannis KN, Panoutsopoulos GI, Demonakou MD, Skaltsas SD, Hereti RI, Papadopoulou-Daifoti Z, Mykoniatis MG. Effect of serotonin receptor 2 blockage on liver regeneration after partial hepatectomy in the rat liver. *Liver Int.*, **26**, 352–361 (2006).
8) Ruddell RG, Mann DA, Ramm GA. The function of serotonin within the liver. *J. Hepatol.*, **48**, 666–675 (2008).
9) Murata S, Maruyama T, Nowatari T, Takahashi K, Ohkouchi N. Signal transduction of platelet-induced liver regeneration and decrease of liver fibrosis. *Int. J. Mol. Sci.*, **15**, 5412–5425 (2014).
10) Porter RHP, Benwell KR, Lamb H, Malcolm CS, Allen NH, Revell DF, Adams DR, Sheardown MJ. Functional characterization of agonists at recombinant human 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors in CHO-K1 cells. *Br. J. Pharmacol.*, **128**, 13–20 (1999).
11) Schmuck K, Ullmer C, Engels P, Lübbert H. Cloning and functional characterization of the human 5-HT_{2B} serotonin receptor. *FEBS Lett.*, **342**, 85–90 (1994).
12) Balasubramanian S, Paulose CS. Induction of DNA synthesis in primary cultures of rat hepatocytes by serotonin: possible involvement of serotonin S_2 receptor. *Hepatology*, **27**, 62–66 (1998).
13) Bonhaus DW, Bach C, DeSouza A, Salazar FH, Matsuoka BD, Zuppan P, Chan HW, Eglen RM. The pharmacology and distribution of human 5-hydroxytryptamine$_{2B}$ (5-HT_{2B}) receptor gene products: comparison with 5-HT_{2A} and 5-HT $_{2C}$ receptors. *Br. J. Pharmacol.*, **115**, 622–628 (1995).
14) Zgombick JM, Schechter LE, Kucharewicz SA, Weinshank RL, Branchek TA. Ketanserin and ritanserin discriminate between recombinant human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor subtypes. *Eur. J. Pharmacol.*, **291**, 9–15 (1995).
15) Audia JE, Evrard DA, Murdoch GR, Droste JJ, Nissen JS, Schenck KW, Fludzinski P, Lucaites VL, Nelson DL, Cohen ML. Potent, selective tetrahydro-*β*-carboline antagonists of the serotonin 2B ($5HT_{2B}$) contractile receptor in the rat stomach fundus. *J. Med. Chem.*, **39**, 2773–2780 (1996).
16) Bromidge SM, Duckworth M, Forbes IT, Ham P, King FD, Thewlis KM, Blaney FE, Naylor CB, Blackburn TP, Kennett GA, Wood MD, Clarke SE. 6-Chloro-5-methyl-1-[[2-[(2-methyl-3-pyridyl)oxy]-5-pyridyl]carbamoyl]-indoline (SB-242084): the first selective and brain penetrant 5-HT2C receptor antagonist. *J. Med. Chem.*, **40**, 3494–3496 (1997).
17) Seglen PO. Preparation of isolated liver cells. *Methods Cell Biol.*, **13**, 29–83 (1976).
18) Kimura M, Ogihara M. Density-dependent proliferation of adult rat hepatocytes in primary culture induced by epidermal growth factor is potentiated by cAMP-elevating agents. *Eur. J. Pharmacol.*, **324**, 267–276 (1997).
19) Morley CGD, Kingdon HS. Use of ^{3}H-thymidine for measurement of DNA synthesis in rat liver-a warning. *Anal. Biochem.*, **45**, 298–305 (1972).
20) Lees MB, Paxman S. Modification of the Lowry procedure for the analysis of proteolipid protein. *Anal. Biochem.*, **47**, 184–192 (1972).
21) Okamoto H, Kimura M, Watanabe N, Ogihara M. Tumor necrosis factor (TNF) receptor-2-mediated DNA synthesis and proliferation in primary cultures of adult rat hepatocytes: The involvement of endogenous transforming growth factor-*α*. *Eur. J. Pharmacol.*, **604**, 12–19 (2009).
22) Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970).
23) Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350–4354 (1979).
24) Kimura M, Okamoto H, Ogihara M. Activation of mitogen-activated protein kinase by hepatocyte growth factor is stimulated by both alpha1- and beta2-adrenergic agonists in primary cultures of adult rat hepatocytes. *J. Pharmacol. Sci.*, **103**, 398–407 (2007).
25) Kennett GA, Bright F, Trail B, Baxter GS, Blackburn TP. Effects of the 5-HT_{2B} receptor agonist, BW723C86, on the three rat model of anxiety. *Br. J. Pharmacol.*, **117**, 1443–1448 (1996).
26) McLean TH, Parrish JC, Braden MR, Marona-Lewicka D, Gallardo-Godoy A, Nichols DE. 1-Aminomethylbenzocycloalkanes: conformationally restricted hallucinogenic phenethylamine analogues as functionally selective 5-HT_{2A} receptor agonists. *J. Med. Chem.*, **49**, 5794–5803 (2006).
27) Kalgutkar AS, Dalvie DK, Aubrecht J, Smith EB, Coffing SL, Cheung JR, Vage C, Lame ME, Chiang P, McClue KF, Maurer TS, Coelho RV Jr, Soliman VF, Schildknegt K. Genotoxicity of 2-(3-chlorobenzyloxy)-6-(piperazinyl)piperazine, a novel 5-hydroxytryptamine$_{2C}$ receptor agonist for the treatment of obesity: role of metabolic activation. *Drug Metab. Dispos.*, **35**, 848–858 (2007).
28) Levitzki A, Gazit A. Tyrosine kinase inhibition: an approach to drug development. *Science*, **267**, 1782–1788 (1995).
29) Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4*H*-l-benzopyran-4-one (LY294002). *J. Biol. Chem.*, **269**, 5241–5248 (1994).
30) Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.*, **270**, 27489–27494 (1995).
31) Davis RJ. The mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem.*, **268**, 14553–14556 (1993).
32) Chung J, Kuo CJ, Crabtree GR, Blenis J. Rapamycin-FKBP specifically blocks growth-dependent activation of the signalling by the 70 kDa S6 protein kinases. *Cell*, **69**, 1227–1236 (1992).
33) Thompson AK, Mostafapour SP, Denlinger LC, Bleasdale JE, Fisher SK. The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. *J. Biol. Chem.*, **266**, 23856–23862 (1991).
34) Szabo E, Qiu Y, Baksh S, Michalak M, Opas M. Calreticulin inhibits commitment to adipocyte differentiation. *J. Cell Biol.*, **182**, 103–116 (2008).
35) Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D, Kirilovsky J. The bisindolylmaleimide GF109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.*, **266**, 15771–15781 (1991).
36) Zuscik MJ, Puzas JE, Rosier RN, Gunter KK, Gunter TE. Cyclic-AMP-dependent protein kinase activity is not required by parathyroid hormone to stimulate phosphoinisitide signaling in chondrocytes but is required to transduce the hormone's proliferative effect. *Arch. Biochem. Biophys.*, **315**, 352–361 (1994).
37) Holgate ST, Lewis RA, Austen KF. Role of adenylate cyclase in immunologic release of mediators from rat mast cells: agonist and antagonist effects of purine- and ribose-modified adenosine analogs. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 6800–6804 (1980).
38) Kimura M, Osumi S, Ogihara M. Prostaglandin E_2 (EP_1) receptor agonist-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes: the involvement of TGF-*α*. *Endocrinology*, **142**, 4428–4440 (2001).
39) Kimura M, Okamoto H, Natsume H, Ogihara M. IP receptor agonist-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes: the involvement of endogenous transforming growth factor-*α*. *J. Pharmacol. Sci.*, **109**, 618–629 (2009).