

Ras-dependent ERK Activation by the Human G_s-coupled Serotonin Receptors 5-HT_{4(b)} and 5-HT_{7(a)}*

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Receptor tyrosine kinases activate mitogen-activated protein (MAP) kinases through Ras, Raf-1, and MEK. Receptor tyrosine kinases can be transactivated by G protein-coupled receptors coupling to G_i and G_q. The human G protein-coupled serotonin receptors 5-HT_{4(b)} and 5-HT_{7(a)} couple to G_s and elevate intracellular cAMP. Certain G_s-coupled receptors have been shown to activate MAP kinases through a protein kinase A- and Rap1-dependent pathway. We report the activation of the extracellular signal-regulated kinases (ERKs) 1 and 2 (p44 and p42 MAP kinase) through the human serotonin receptors 5-HT_{4(b)} and 5-HT_{7(a)} in COS-7 and human embryonic kidney HEK293 cells. In transfected HEK293 cells, 5-HT-induced activation of ERK1/2 is sensitive to H89, which indicates a role for protein kinase A. The observed activation of ERK1/2 does not require transactivation of epidermal growth factor receptors. Furthermore, 5-HT induced activation of both Ras and Rap1. Whereas the presence of Rap1GAP1 did not influence the 5-HT-mediated activation of ERK1/2, the activation of ERK1/2 was abolished in the presence of dominant negative Ras (RasN17). ERK1/2 activation was reduced in the presence of “dominant negative” Raf1 (RafS621A) and slightly reduced by dominant negative B-Raf, indicating the involvement of one or more Raf isoforms. These findings suggest that activation of ERK1/2 through the human G_s-coupled serotonin receptors 5-HT_{4(b)} and 5-HT_{7(a)} in HEK293 cells is dependent on Ras, but independent of Rap1.

Extracellular signals such as growth factors and hormones regulate cell proliferation. In general, growth factors activate receptor tyrosine kinases (RTKs),¹ whereas most hormones

activate G protein-coupled receptors (GPCRs). Intracellular cascades such as the mitogen-activated protein (MAP) kinase, also called the extracellular signal-regulated kinase (ERK), cascade, are mainly activated as a consequence of agonist stimulation of RTKs. ERK1/2 are serine/threonine protein kinases activated upon dual phosphorylation by the MAP kinase kinase MEK (1), which is phosphorylated and activated by MAP kinase kinase kinases of the Raf family (2). In mammals, all three Raf isoforms expressed (A-Raf, B-Raf, and Raf-1) are cytosolic in resting cells and become activated upon recruitment to the plasma membrane by activated, small G proteins of the Ras family (H-Ras, K-Ras, N-Ras, and Rap1). Ras, in its active, GTP-bound conformation, may activate all isoforms of Raf. The levels of GTP-bound Ras are controlled by the actions of guanine nucleotide exchange factors and GTPase-activating proteins (GAPs). The two major Ras-specific guanine nucleotide exchange factors are Son of sevenless (SOS) (3) and Ras-GRF1 (CDC25^{Mm}) (4). SOS is mainly thought to activate Ras as a consequence of RTK stimulation, whereas Ras-GRF1 activates Ras in response to Ca²⁺ signaling and GPCR-mediated signals (5–8).

Serotonin (5-hydroxytryptamine (5-HT)) mediates its diverse physiological effects through at least 14 different receptors, of which 13 are GPCRs or so-called seven transmembrane-spanning receptors (9). The 5-HT receptors, each encoded by a separate gene, have been grouped into families called 5-HT₁ to 5-HT₇, with some of the families containing several members. The human serotonin receptors 5-HT₄ and 5-HT₇ couple to the heterotrimeric G protein G_s and exist in multiple splice variants (10, 11). Stimulation of these receptors leads to activation of adenylyl cyclase and a rapid increase in the formation of the intracellular second messenger cAMP. Elevated levels of cAMP have several intracellular effects, e.g. activation of cAMP-dependent protein kinase (PKA) and exchange proteins directly activated by cAMP (Epacs), guanine nucleotide exchange factors specific for Rap. Both mechanisms have been claimed to be involved in cAMP-dependent activation of Rap1 (12–14).

The main target for cAMP is PKA, which has cell type-specific effects on the ERK1/2 cascade. In several cell types, Ras-dependent ERK1/2 activation is antagonized by PKA (15–18). In other cell types, PKA appears to activate the ERK1/2 cascade via Rap1 and B-Raf (12, 19). In human embryonic kidney HEK293 cells, isoproterenol stimulation of the endogenous G_s-coupled β_2 -adrenergic receptor (β_2 -AR) activates Ras and Rap1, but only Rap1 is able to mediate activation of ERK1/2 by recruiting B-Raf to the membrane and thereafter activate MEK (20). However, at overexpressed levels of β_2 -ARs, ERK1/2 can be activated through the G $\beta\gamma$ subunits of pertussis toxin-sensitive G proteins, interpreted

lular signal-regulated kinase kinase; Epac, exchange protein directly activated by cAMP; SH, Src homology.

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¹ The abbreviations used are: RTK, receptor tyrosine kinase; 5-HT, 5-hydroxytryptamine (serotonin); β_2 -AR, β_2 -adrenergic receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GAP, GTPase-activating protein; GST, glutathione S-transferase; GPCR, G protein-coupled receptor; MAP, mitogen-activated protein; PKA, protein kinase A; RBD, Ras-binding domain; MEK, mitogen-activated protein kinase/extracel-

as a switch in coupling from G_s to G_i due to phosphorylation of the β_2 -AR by PKA (21).

Upon stimulation of G_i - (22) and G_q -coupled (23) receptors, activation of MAP kinases is induced by $G\beta\gamma$ subunits that mediate Ras activation through proteins such as the tyrosine kinase c-Src, Grb2, and SOS (24). Both phosphoinositide 3-kinase γ (25) and the calcium-sensitive kinase PYK2 (26) have been implicated in the activation of c-Src. The $G\alpha_i$ and $G\alpha_q$ subunits may also mediate ERK1/2 activation by binding to Rap1GAP1, a selective Rap1 GTPase-activating protein (27, 28). In these cases, active Rap1 antagonizes the function of Ras, and inhibition of Rap1 by Rap1GAP1 allows Ras to signal to ERK1/2. Furthermore, in several cell types, GPCRs coupling to G_i and G_q have been reported to transactivate the epidermal growth factor receptor (EGFR) and thereby lead to activation of ERK1/2 (29–31).

A G_s -coupled 5-HT receptor has been implicated in ERK activation and LTP induction in *Aplysia* (32, 33). In mouse cortical neurons, several monoamines including 5-HT, as well as forskolin, activated ERK1/2 and increased the level of GTP-bound Ras, but the 5-HT receptor involved was not characterized (34). In cultured rat hippocampal neurons, 5-HT activated ERK1/2 through 5-HT₇ receptors, but the mechanism was not characterized (35). In mammalian brain, both 5-HT₄ and 5-HT₇ receptors are found in the hippocampus (36, 37), and these receptors are both believed to be important in learning and memory (38). Thus, there is a need to determine whether 5-HT₄ and 5-HT₇ receptors can activate ERK, as well as the mechanism for such activation. We have previously characterized activation of adenylyl cyclase by different splice variants of the human G_s -coupled 5-HT₄ (39) and 5-HT₇ (40) receptors. We now demonstrate that both the human G_s -coupled serotonin receptors 5-HT_{4(b)} and 5-HT_{7(a)}, when transiently expressed in HEK293 and COS-7 cells, activate ERK1/2 through a mechanism dependent on Ras and independent of Rap1.

EXPERIMENTAL PROCEDURES

Materials—HEK293 and COS-7 cells were from American Type Culture Collection (Manassas, VA). Mouse monoclonal anti-phospho-ERK1/2 was from Cell Signaling Technology (Beverly, MA), sheep polyclonal anti-mouse Ig-horseradish peroxidase and sheep anti-rabbit IgG-horseradish peroxidase were from Amersham Biosciences, rabbit polyclonal anti-ERK1/2 was from Upstate Biotechnology (Lake Placid, NY), mouse monoclonal anti-Ras (IgG₁) was from Transduction Laboratories (BD Biosciences), and rabbit polyclonal anti-Raf-1 and rabbit polyclonal anti-B-Raf were from Santa Cruz Biotechnology (Santa Cruz, CA). 5-HT hydrochloride (serotonin), *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrobromide (H89), epidermal growth factor (EGF), Dulbecco's modified Eagle's medium, and diethylaminoethyl-dextran hydrochloride (DEAE-dextran) were from Sigma. PD153035 was from Tocris Cookson Ltd. (Avonmouth, UK), and PD98059 was from Calbiochem. Glutathione-Sepharose 4B and Hybond-P (polyvinylidene difluoride) membrane were from Amersham Biosciences. LipofectAMINE™ reagent and penicillin-streptomycin were from Invitrogen. UltraCULTURE™ general purpose serum-free medium and L-glutamine were from BioWhittaker Europe (Verviers, Belgium). Bio West extended duration chemiluminescent substrate was from UVP (Upland, CA), and the BC assay protein quantitation kit was from Uptima (Monticon, France).

Plasmids—The pcDNA3.1(–) vectors encoding the human 5-HT_{4(b)} and 5-HT_{7(a)} receptors were described previously (39, 40). The pcDNA3.1 vector encoding the dominant negative Rap1, RapN17, was provided by Dr. Philip J. S. Stork (Oregon). Rap1GAP1, GST-Raf1-RBD, and GST-RalGDS-RBD constructs were provided by Dr. Johannes L. Bos (Utrecht, The Netherlands) (41, 43). The pCMV vectors encoding the dominant negative Ras, RasN17, constitutively active Ras, RasV12, dominant negative Raf1, RafS621A, and constitutively active Raf1, Raf-CAAX, were from Clontech. The pcDNA3.1 vectors encoding the dominant negative B-Raf, N-B-Raf, and constitutively active B-Raf, CAAX-B-Raf, were provided by Dr. Roser Buscà (Nice, France) and Dr. Alain Eyche (Orsay, France) (42).

Cell Culture and Transfection—COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum

and 50 mg/liter gentamicin at 37 °C in a humidified atmosphere of 5% CO₂ in air and transfected at 60–70% confluence with the 5-HT_{4(b)} or 5-HT_{7(a)} receptors using DEAE-dextran according to the manufacturer's protocol. HEK293 cells were cultured in UltraCULTURE™ general purpose serum-free medium with 2 mM L-glutamine and 100 units/ml penicillin-streptomycin at 37 °C in a humidified atmosphere of 5% CO₂ in air and transfected at 60–70% confluence with the indicated cDNA(s) using LipofectAMINE according to the manufacturer's protocol. When necessary, empty vector (pcDNA3.1(–); Invitrogen) was included to assure that each dish received the same amount of DNA (1.0 and 2.9 μ g of plasmid DNA per 35-mm and 60-mm dish, respectively). If not otherwise indicated, cells were kept in serum-free medium as described until treatment and lysis, ~48 h after transfection. When indicated, cells were preincubated with 20 μ M H89 for 25 min, 5 μ M PD98059 for 20 min, or 1 μ M PD153035 for 18 h. Cells were stimulated with agonist (10 μ M 5-HT or 5 nM EGF) for 5 min, if not otherwise indicated. All experiments were carried out in doublets with at least three replicates, if not otherwise indicated.

Western Blotting—Equal amounts of cell lysate proteins were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies (anti-phospho-ERK1/2, 1:2,000; anti-Ras, 1:1,000; anti-Rap1, 1:500) in 5% nonfat dry milk in phosphate-buffered saline-0.05% Tween and thereafter incubated with corresponding horseradish peroxidase-conjugated secondary antibodies. The immobilized horseradish peroxidase-conjugated secondary antibodies were visualized with Bio West chemiluminescent substrate and analyzed with a UVP BioChemie system. After detection of phospho-ERK1/2, membranes were stripped by treatment with 0.5 M NaOH for 5 min at room temperature and extensive washing with distilled H₂O and reprobed with anti-ERK1/2 antibodies (1:10,000) to confirm equal loading.

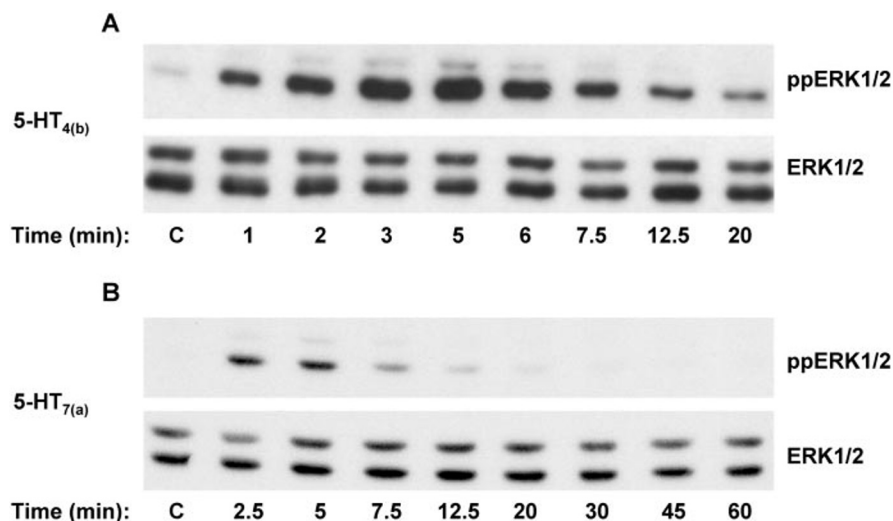
Phospho-ERK1/2 Assay—The cells were cultured in 35-mm dishes, transfected and stimulated with agonist as described, lysed in ice-cold cell lysis buffer (1% SDS, 1 mM Na₃VO₄, 50 mM Tris-HCl, pH 7.4 at room temperature), scraped with a rubber policeman, sheared through a 25-gauge syringe, and immediately snap frozen in liquid N₂. The thawed cell lysates were cleared at 13,000 \times g at 4 °C, and the protein concentrations in the supernatants were quantified using the BC assay protein quantitation kit using BSA as standard. Equal amounts of protein were prepared for separation by SDS-PAGE.

Pull-Down Experiments in HEK293 Cells—The cells were assayed as described by van Triest *et al.* (43). Briefly, HEK293 cells were maintained in 60-mm dishes, transfected, and stimulated as described. The cells were lysed in ice-cold pull-down lysis buffer (10% glycerol, 1% Nonidet P-40, 50 mM Tris-HCl, pH 7.5 at room temperature, 200 mM NaCl, 2 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 10 μ g/ml trypsin inhibitor). Equal amounts of cleared cell lysates were incubated at 4 °C on a tumbler for 1 h with GST-Raf1-RBD or GST-RalGDS-RBD constructs precoupled to glutathione-Sepharose beads for Ras or Rap1 pull-down experiments, respectively. The beads were pelleted and washed four times with ice-cold pull-down lysis buffer. The proteins were eluted from the beads by boiling for 5 min in 1 \times loading buffer. The protein samples were loaded and resolved on 12.5% SDS-polyacrylamide gels. Western blotting was performed as described.

RESULTS

ERK1/2 Activation via 5-HT_{4(b)} and 5-HT_{7(a)} Receptors in COS-7 and HEK293 Cells—Initial experiments indicated that treatment of COS-7 cells transiently transfected with G_s -coupled human 5-HT_{4(b)} or 5-HT_{7(a)} receptors with 10 μ M 5-HT induced dual phosphorylation and thus activation of ERK1/2. Similar results were obtained with HEK293 cells. Because HEK293 cells do not express endogenous 5-HT receptors and had a lower background of ERK1/2 phosphorylation, these cells were selected for further study. Based on the potency of 5-HT to stimulate adenylyl cyclase activity through human 5-HT_{4(b)} (39) or 5-HT_{7(a)} (40) receptors and 5-HT concentration-response experiments with ERK1/2 phosphorylation (data not shown), a concentration of 10 μ M 5-HT was chosen for the remaining experiments. A high resolution time course showed that the maximal 5-HT-induced phosphorylation of ERK1/2 through 5-HT_{4(b)} receptors occurred after 5 min (Fig. 1A). Time courses with more extended periods of stimulation showed that 5-HT-induced activation of ERK1/2 through 5-HT_{4(b)} (data not

FIG. 1. Activation of ERK1/2 through 5-HT_{4(b)} and 5-HT_{7(a)} receptors. Time courses of activation of ERK1/2 after 5-HT stimulation of HEK293 cells transiently transfected with the human 5-HT_{4(b)} (A) or 5-HT_{7(a)} (B) receptor. The cells were stimulated for the indicated periods of time and assayed for detection of phospho-ERK1/2 as described under "Experimental Procedures." Proteins were separated on 10% SDS-PAGE and electroblotted over to polyvinylidene difluoride membranes before incubation with antibodies. A and B show representative Western blots probed with phospho-specific ERK1/2 antibodies (*ppERK1/2*; top panels) and subsequently probed with anti-ERK1/2 antibodies (*ERK1/2*; bottom panels) to confirm equal loading.



shown) and 5-HT_{7(a)} (Fig. 1B) receptors peaked after ~5 min and then decreased. There was no second peak or sustained activation of ERK1/2.

Activation of ERK1/2 through 5-HT_{4(b)} and 5-HT_{7(a)} Receptors Is PKA- and MEK-dependent—All splice variants of the human serotonin receptors 5-HT₄ and 5-HT₇ are G_s-coupled receptors that upon agonist stimulation induce a rapid increase in the formation of cAMP, leading to activation of PKA or Epac. To examine the role of PKA in the signaling pathway, HEK293 cells transiently transfected with either 5-HT_{4(b)} or 5-HT_{7(a)} were preincubated with the PKA inhibitor H89. Pretreatment with H89 led to a partial inhibition of 5-HT-induced activation of ERK1/2, as shown in Fig. 2A for the 5-HT_{4(b)} receptor. However, a more efficient inhibition was observed when the transiently transfected cells were maintained in serum-free Dulbecco's modified Eagle's medium for 16–18 h before treatment with 5-HT, as shown in Fig. 2B for the 5-HT_{7(a)} receptor. MEK plays a central role in the classical signaling pathway from cell surface receptors to ERK1/2. Pretreatment with the selective MEK inhibitor PD98059, as described under "Experimental Procedures," blocked 5-HT-induced activation of ERK1/2 in cells transfected with 5-HT_{4(b)} receptors (data not shown) or 5-HT_{7(a)} (Fig. 2C). Taken together, the above data show that 5-HT-induced activation of ERK1/2 by human 5-HT_{4(b)} and 5-HT_{7(a)} receptors is mediated through a pathway dependent on MEK and at least partially dependent on PKA.

Activation of ERK1/2 through 5-HT_{4(b)} and 5-HT_{7(a)} Receptors Does Not Require EGFR Phosphorylation—Several studies have shown that G_s-coupled receptors may transactivate RTKs (29–31). Transiently transfected HEK293 cells were preincubated with the EGFR kinase inhibitor PD153035 to determine possible involvement of EGFR transactivation. The presence of PD153035 did not influence the 5-HT-induced activation of ERK1/2 through 5-HT_{4(b)} (data not shown) or 5-HT_{7(a)} receptors (Fig. 3A). As a positive control, the presence of PD153035 was shown to completely eliminate activation of ERK1/2 by EGF in nontransfected HEK293 cells (Fig. 3B). The above data suggest that activation of ERK1/2 through 5-HT_{4(b)} and 5-HT_{7(a)} receptors does not require transactivation of EGFRs.

5-HT-induced Activation of ERK1/2 Is Independent of Rap1—Activation of ERK1/2 by endogenous β_2 -ARs in HEK293 cells has been reported to be PKA-dependent and mediated through a pathway independent of Ras. Although both Ras and Rap1 were activated by isoproterenol treatment, only Rap1 was capable of coupling to a Raf isoform and activating ERK1/2 (20). The Rap1 interfering mutant, Rap1N17, and the Rap1-specific GTPase-activating protein, Rap1GAP1, were used to

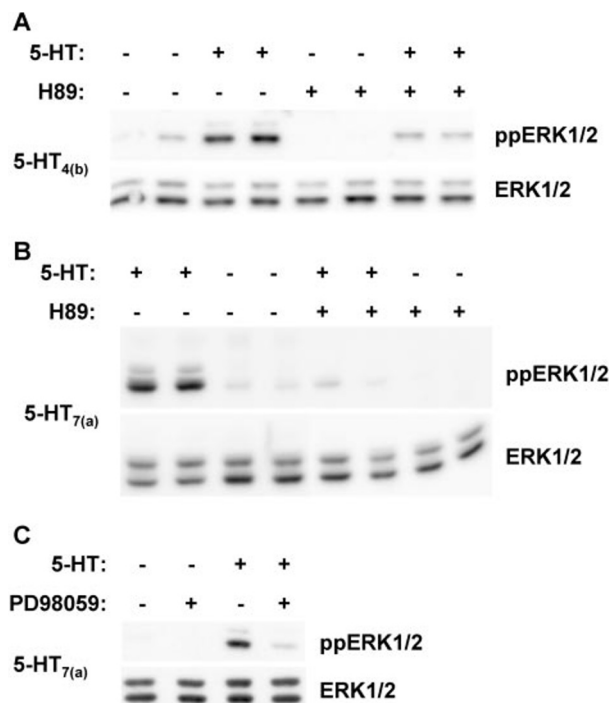


FIG. 2. Activation of ERK1/2 is partly blocked by a PKA inhibitor and dependent on MEK. A and B, HEK293 cells transiently transfected with 5-HT_{4(b)} (A) or 5-HT_{7(a)} (B) were treated with 5-HT or vehicle in the absence or presence of the PKA inhibitor H89 (20 μ M), as indicated. The cells were harvested, and Western blotting was performed as described under "Experimental Procedures." C, HEK293 cells transiently transfected with 5-HT_{7(a)} were pretreated with the MEK inhibitor PD98059 (5 μ M) or vehicle before treatment with 5-HT or vehicle. Representative Western blots shown in A–C were probed as described in Fig. 1.

investigate the role of Rap1 in the activation of ERK1/2 through 5-HT_{4(b)} and 5-HT_{7(a)} receptors in co-transfection experiments. The presence of Rap1N17 (data not shown) or Rap1GAP1 (Fig. 4A) did not influence the 5-HT-induced activation of ERK1/2, although activation of Rap1 was abolished, as evidenced by pull-down of activated Rap1 (Rap1-GTP) with the RBD of RalGDS (Fig. 4B).

5-HT Induces Ras Activation through 5-HT_{4(b)} and 5-HT_{7(a)} Receptors—GPCRs may activate the small GTPase Ras, which plays a central role in the activation of ERK1/2 through several different types of receptors. Therefore, the RBD of Raf1, which only binds activated (GTP-bound) Ras, was used in pull-down

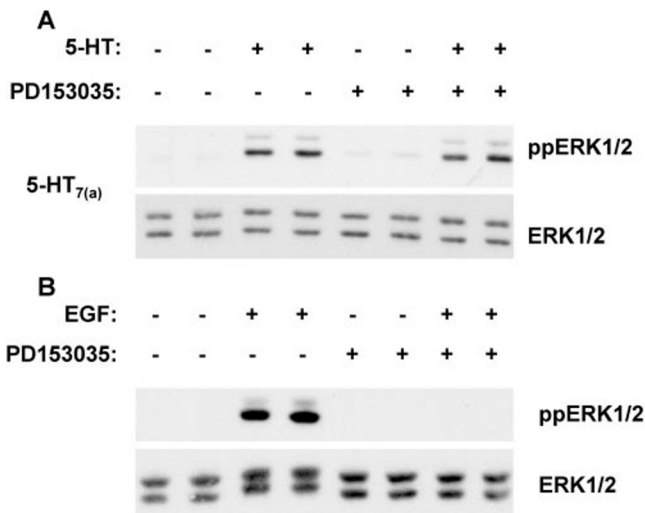


FIG. 3. Activation of ERK1/2 through 5-HT_{7(a)} receptors does not require EGFR phosphorylation. A, HEK293 cells transiently transfected with 5-HT_{7(a)} receptors were treated with 5-HT (10 μ M) or vehicle in the absence or presence of PD153035 (1 μ M) as indicated. B, nontransfected HEK293 cells were treated with EGF (5 nM) or vehicle in the absence or presence of PD153035. Representative Western blots probed as described in Fig. 1 are shown in A and B.

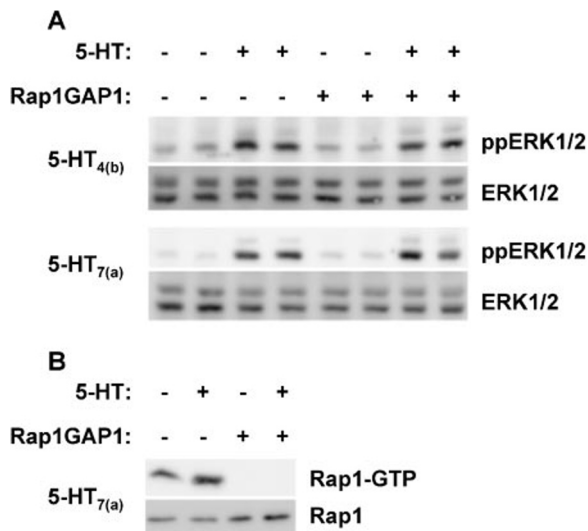


FIG. 4. Activation of ERK1/2 through 5-HT_{4(b)} and 5-HT_{7(a)} receptors is independent of Rap1. HEK293 cells were transiently co-transfected with 5-HT_{4(b)} (A, top panels) or 5-HT_{7(a)} (A, bottom panels, and B) and Rap1GAP1 or empty vector (pcDNA3.1) and treated with 10 μ M 5-HT or vehicle for 5 min. A, representative Western blots, probed as described in Fig. 1, are shown. B, the Ras-binding domain of RalGDS was used to pull down activated Rap1 (Rap1-GTP) from cell lysates, as described under "Experimental Procedures." Representative Western blots probed with anti-Rap1 are shown to illustrate activated (Rap1-GTP, top panel) and total (Rap1, bottom panel) Rap1. Note that the presence of Rap1GAP1 completely eliminated activation of Rap1.

experiments to test whether 5-HT could induce activation of Ras. Such experiments documented activation of Ras by 5-HT through both 5-HT_{4(b)} receptors (data not shown) and 5-HT_{7(a)} receptors (Fig. 5A). Nontransfected HEK293 cells were treated with EGF as a positive control for Ras activation (Fig. 5A).

Activation of ERK1/2 through 5-HT_{4(b)} and 5-HT_{7(a)} Receptors Is Ras-dependent—Dominant negative and constitutively active mutants of Ras were used to further investigate the role of Ras in the 5-HT-induced activation of ERK1/2. Activation of ERK1/2 through 5-HT_{4(b)} and 5-HT_{7(a)} receptors was highly reduced in the presence of the dominant negative variant of Ras, RasN17 (Fig. 5B). The data in Figs. 4 and 5 suggest that

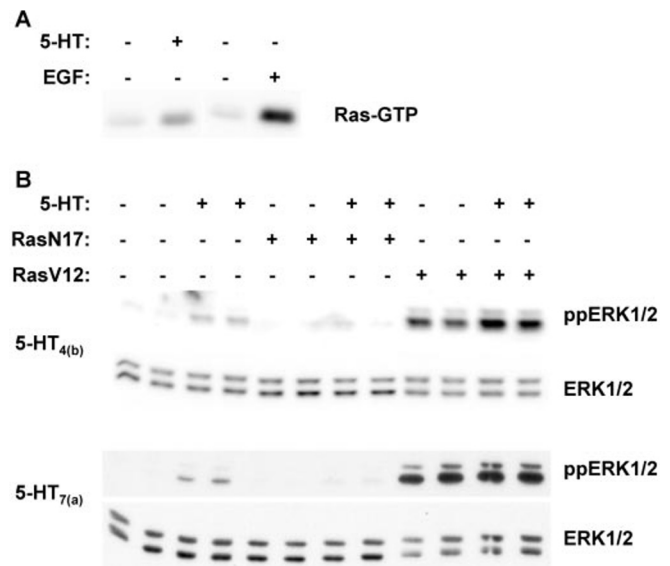


FIG. 5. Activation of ERK1/2 through 5-HT_{4(b)} and 5-HT_{7(a)} receptors is dependent on Ras. A, Ras pull-down experiments were performed after stimulating 5-HT_{7(a)} receptor-transfected HEK293 cells with vehicle or 5-HT (10 μ M) or nontransfected HEK293 cells with vehicle or EGF (5 nM). Pull-down experiments were carried out in single-dish experiments and repeated at least three times. A representative Western blot probed with anti-Ras antibodies is shown. B, HEK293 cells co-transfected with 5-HT_{4(b)} (top panels) or 5-HT_{7(a)} (bottom panels) and empty vector, RasN17, or RasV12 were stimulated with vehicle or 5-HT (10 μ M) for 5 min. For each receptor, the panels in B show representative Western blots probed as described in Fig. 1.

the 5-HT-induced activation of ERK1/2 is dependent on Ras, but independent of Rap1. When cells co-transfected with 5-HT_{4(b)} receptors and the constitutively active variant of Ras, RasV12, were treated with 5-HT, an additional activation of ERK1/2 was observed compared with the constitutive activation of ERK1/2 in these cells.

Raf Interfering Mutants Inhibit 5-HT-induced Activation of ERK1/2—HEK293 cells express several effectors of activated Ras, e.g. different isoforms of Raf. HEK293 cells were co-transfected with a point-mutated variant of Raf1, RafS621A, which interferes with Raf1-mediated signaling, or with the constitutively active variant RafCAAX to investigate the role of Raf1 in the activation of ERK1/2 through 5-HT_{4(b)} and 5-HT_{7(a)} receptors. The cells were incubated for 16–18 h in serum-free Dulbecco's modified Eagle's medium before stimulation with 5-HT, as described under "Experimental Procedures." Expression of RafS621A suppressed the activation of ERK1/2 mediated through 5-HT_{4(b)} (Fig. 6A, top panels) and 5-HT_{7(a)} (Fig. 6A, bottom panels) receptors. When cells co-transfected with RafCAAX and 5-HT_{4(b)} (data not shown) or 5-HT_{7(a)} (Fig. 6B) receptors were treated with 5-HT, increased phosphorylation of ERK1/2 was observed compared with the constitutive ERK1/2 phosphorylation in these cells.

To determine the role of B-Raf, HEK293 cells were co-transfected with 5-HT_{7(a)} receptors and dominant negative (N-B-Raf) or constitutively active (CAAX-B-Raf) B-Raf. The dominant negative mutant of B-Raf corresponds to the N-terminal region of quail B-Raf (amino acids 1–443) (42). In several experiments, the presence of N-B-Raf had only a weak inhibitory effect on 5-HT-induced activation of ERK1/2 (Fig. 7A). In general, the inhibitory effect of N-B-Raf was weaker than that of RafS621A. Additional activation of ERK1/2 was induced by 5-HT in the presence of CAAX-B-Raf, as was the case with RafCAAX (Fig. 7A). Overexpression of RafS621A and N-B-Raf was verified by Western blot analysis using anti-Raf1 (Fig. 6C) and anti-B-Raf (Fig. 7B) antibodies, respectively.

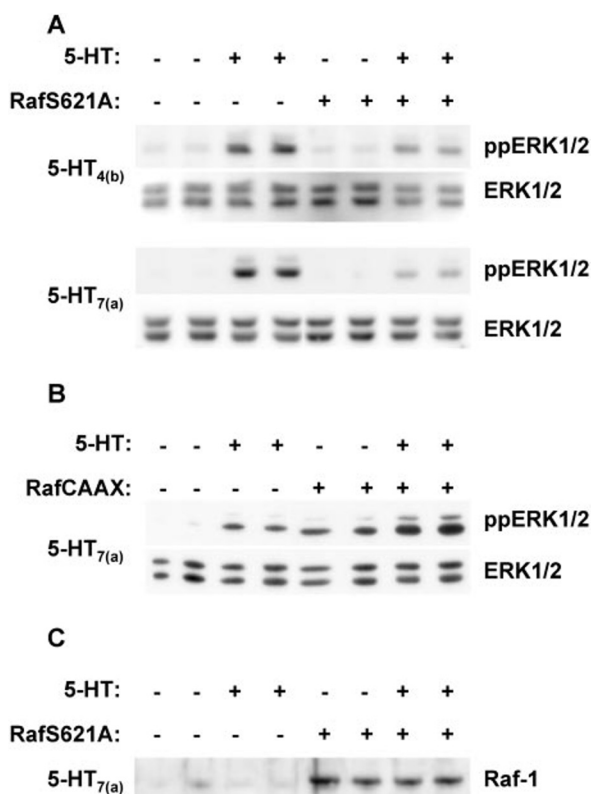


FIG. 6. Raf-1 interfering mutants reduce the 5-HT-stimulated activation of ERK1/2. *A*, HEK293 cells co-transfected with 5-HT_{4(b)} (top panels) or 5-HT_{7(a)} (bottom panels) receptor and the interfering Raf1 mutant RafS621A or empty pcDNA3.1 vector as control were stimulated and harvested for phospho-ERK1/2 assay as described under "Experimental Procedures." *B*, HEK293 cells were transiently co-transfected with the 5-HT_{7(a)} receptor and constitutively active Raf1 (RafCAAX) or empty vector as control and stimulated with vehicle or 5-HT (10 μ M) for 5 min. The Western blots shown in *A* and *B* were probed as described in Fig. 1. *C*, overexpression of RafS621A in HEK293 cells co-transfected with 5-HT_{7(a)} receptors and RafS621A was confirmed by incubating Western blots with anti-Raf-1. The interfering mutant RafS621A is clearly overexpressed compared with endogenous levels of Raf-1 in cells transfected without RasS621A.

DISCUSSION

In this paper, we report the activation of ERK1/2 by 5-HT through the human G_s-coupled serotonin receptors 5-HT_{4(b)} and 5-HT_{7(a)} transiently expressed in COS-7 and HEK293 cells. We also demonstrate that the pathway in HEK293 cells involves Ras but not Rap1. Pretreatment of transiently transfected HEK293 cells with H89 suppressed 5-HT-induced dual phosphorylation of ERK1/2, indicating a role for PKA.

Intracellular signals from the large family of G protein-coupled receptors are conveyed by α subunits (G α) or β and γ subunits (G $\beta\gamma$) of heterotrimeric G proteins. Of the four main classes of heterotrimeric G proteins (G_s, G_i, G_q, and G_{12/13}), G_q- and G_i-coupled receptors have been shown to mediate activation of ERK1/2 through free G $\beta\gamma$ subunits (44, 45). G $\beta\gamma$ subunits released upon stimulation of G_s-coupled receptors have not been shown to mediate activation of ERK1/2, possibly because cells contain far less G_s than G_i or G_q. However, G α_s activates adenylyl cyclase and thereby PKA, which has been proposed to play an essential role in the ERK1/2 cascade (12, 20, 34). In line with this, we found that ERK1/2 activation through the G_s-coupled receptors 5-HT_{4(b)} and 5-HT_{7(a)} was inhibited in cells pretreated with the PKA inhibitor H89.

In addition to activation of PKA, cAMP may lead to activation of Rap1 through the Rap1-selective exchange factors Epac1 or Epac2 (13, 14, 46). The small GTPase Rap1 is a Ras

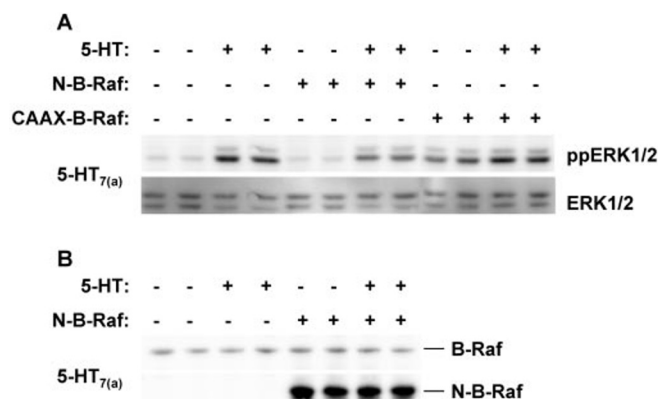


FIG. 7. Dominant negative B-Raf has little effect on 5-HT-stimulated activation of ERK1/2. HEK293 cells co-transfected with 5-HT_{7(a)} receptor and the dominant negative B-Raf construct N-B-Raf or the constitutively active B-Raf construct CAAX-B-Raf or empty vector as control were stimulated and harvested for phospho-ERK1/2 assay as described under "Experimental Procedures." *A*, the presence of N-B-Raf barely reduced the 5-HT-mediated activation of ERK1/2. Treatment of HEK293 cells co-transfected with 5-HT_{7(a)} receptor and CAAX-B-Raf with 5-HT caused additional phosphorylation of ERK1/2 compared with the constitutive level of activation of ERK1/2 in these cells. *B*, overexpression of N-B-Raf (N-terminal amino acids 1–443) was confirmed by incubating Western blots with anti-B-Raf.

analogue, which upon isoproterenol stimulation of HEK293 cells has been reported to recruit B-Raf to the membrane and thereby activate the ERK1/2 cascade (20). We determined the role of Rap1 in the observed 5-HT-induced activation of ERK1/2 by co-transfecting HEK293 cells with either Rap1GAP1 or Rap1N17 and 5-HT receptors. Even though Rap1N17 is not a true dominant negative (47, 48), it has been used to inhibit Rap1 activity in several studies (12, 20, 49). Therefore, we used Rap1N17 to support the data obtained with Rap1GAP1. The observed 5-HT-induced activation of ERK1/2 in HEK293 cells was not influenced by co-expression of either Rap1GAP1 or Rap1N17, although Rap1 activation was eliminated. Thus, the G_s-coupled 5-HT_{4(b)} and 5-HT_{7(a)} receptors mediate activation of ERK1/2 through a Rap1-independent pathway, in contrast to what was reported for β_2 -ARs (20).

The small GTPase Ras is the best characterized and serves as the main activator of the ERK1/2 cascade by linking RTKs and all isoforms of Raf. RTK-mediated activation of Ras involves a series of SH2- and SH3-dependent protein-protein interactions between tyrosine-phosphorylated receptor, the adapter proteins Shc and Grb2, and the Ras-guanine nucleotide exchange factor SOS (50, 51). Certain GPCRs coupling to G_i and G_q mediate activation of Ras through free G $\beta\gamma$ subunits, e.g. by stimulation of Shc (44, 45, 52). Isoproterenol stimulation of endogenous β_2 -ARs in HEK293 cells was reported to activate both Rap1 and Ras, but only Rap1 was implicated in the activation of ERK1/2 (20). The mechanism of Ras activation was not addressed. Stimulation of HEK293 cells transiently transfected with 5-HT_{4(b)} or 5-HT_{7(a)} receptors also increased the amount of GTP-bound Ras, and, in addition, 5-HT-induced activation of ERK1/2 was inhibited by expression of the dominant negative mutant of Ras, RasN17. Thus, activation of ERK1/2 through G_s-coupled 5-HT_{4(b)} and 5-HT_{7(a)} receptors in HEK293 cells is Ras-dependent.

Since the original manuscript was submitted for publication, two papers have been published that support the finding of Rap1-independent activation of ERK1/2 through G_s-coupled receptors (53, 54). Furthermore, in accordance with our finding in HEK293 cells of a Ras-dependent mechanism for activation of ERK1/2, Enserink *et al.* (54) report a Ras-dependent activa-

tion of ERK1/2 in Chinese hamster ovary cells subsequent to elevation of intracellular cAMP.

Transactivation of RTKs has been proposed as an alternative mechanism for activation of Ras through GPCRs (55). The signal will pass the membrane three times, and activation of Ras and the ERK1/2 cascade will subsequently take place as by direct stimulation of RTKs, *e.g.* EGFR. Activation of ERK1/2 through 5-HT_{4(b)} and 5-HT_{7(a)} receptors was not inhibited in the presence of the EGFR kinase inhibitor PD153035, indicating that EGFR transactivation is not required.

The effects of cAMP on the ERK1/2 cascade are cell type-specific. In some cell types, PKA was found to inhibit RTK-mediated activation of ERK1/2 (15–18). Raf is the main target for cAMP-mediated regulation of the ERK1/2 cascade. The serine residues 43, 259, and 621 have all been suggested to be involved in the regulation of Raf1 activity (56). Phosphorylation of serine 43 does not inhibit the catalytic activity of Raf1, but it has been proposed to disrupt the normal Ras-Raf association and Raf1 activation (15). Furthermore, even though B-Raf does not have a phosphorylation site analogous to serine 43 in Raf1, elevation of intracellular cAMP levels by forskolin inhibited the EGF-dependent activation of B-Raf (57). The serine 621 residue of Raf1 has been suggested as PKA phosphorylation site. Raf1 phosphorylated at this site exhibited reduced affinity for activated Ras as well as impaired catalytic activity (58). Furthermore, mutation of serine 621 to alanine resulted in loss of catalytic activity. Thus, a serine 621 to alanine mutant, RafS621A, has been used as a “dominant negative” variant of Raf1. However, recent reports have shown that the list of residues involved in the PKA-dependent regulation of Raf1 activity may also include serine residues 233 and 338 (59, 60). Based on the literature concerning cAMP-mediated regulation of Raf1, RafS621A cannot be considered a true dominant negative. Still, we found that the Ras-dependent, 5-HT-induced activation of ERK1/2 was partly inhibited by co-expression of the Raf1 interfering mutant RafS621A, implicating Raf1 in the pathway. In melanocytes, dominant negative B-Raf has been used to show that Ras and B-Raf were involved in cAMP-mediated activation of ERK1/2 (42). We found only little or no effect of co-expression of dominant negative B-Raf under conditions where RafS621A caused more pronounced reduction of ERK1/2 activation by 5-HT. Whether RafS621A is a true dominant negative or only a partial inhibitor of Raf1 activation, these data suggest that one or more isoforms of Raf are involved in the activation of ERK1/2 through 5-HT_{4(b)} and 5-HT_{7(a)} receptors in HEK293 cells.

The immediate upstream activator of ERK1/2 is MEK (61), which is involved in most pathways described for activation of ERK1/2. The presence of the MEK-specific inhibitor PD98059 inhibited 5-HT-induced activation of ERK1/2 through 5-HT_{4(b)} and 5-HT_{7(a)} receptors. These results suggest that the 5-HT-induced activation of ERK1/2 is MEK-dependent.

The high number of publications concerning activation of MAP kinases via G_s-coupled receptors illustrates the complexity of this field. Several publications have shown that activation of Ras may occur after stimulation of G_s-coupled receptors. However, little is known about how these receptors or agents that elevate intracellular levels of cAMP mediate activation of Ras. Src or a Src-like kinase has been suggested to be involved in the activation of ERK1/2 subsequent to stimulation of the G_s-coupled A_{2A}-adenosine receptor (53). However, this may not account for the 5-HT-induced Ras-dependent activation of ERK1/2 in HEK293 cells because the Src-dependent mechanism was observed in Chinese hamster ovary, PC12, and NIH3T3 cells, but not in HEK293 cells. To our knowledge, a Ras-specific exchange factor activated by cAMP, analogous to

Epac for Rap1, has not been published. This does not mean that a Ras-specific exchange factor directly activated by cAMP or PKA phosphorylation does not exist. Alternatively, there may be more indirect actions of PKA via known or unknown proteins that mediate activation of Ras.

The biological significance of the observed activation of ERK1/2 by 5-HT₄ and 5-HT₇ receptors is unknown. The biological effects of 5-HT are mediated by at least 14 different receptors, and there is hardly a target cell for 5-HT that expresses only one type of 5-HT receptor. Based on current knowledge about signal transduction mechanisms and ERK activation, other 5-HT receptors than 5-HT₄ and 5-HT₇ would be expected to play a more important role in ERK activation. However, examples of biological systems exist where ERK activation by G_s-coupled serotonin receptors may be important. In *Aplysia*, activation of mitogen-activated protein kinases by serotonin is implicated in memory formation and occurs through a G_s-coupled 5-HT receptor (32, 33). In mammalian brain, 5-HT₄ and 5-HT₇ receptors are both found in the hippocampus (36, 37), and 5-HT₇ receptors were recently shown to activate ERKs in cultured hippocampal neurons (35). The importance of ERK activation for the involvement of these receptors in learning and memory (38) remains to be determined.

In conclusion, we demonstrate activation of ERK1/2 by 5-HT through the human G_s-coupled 5-HT_{4(b)} and 5-HT_{7(a)} receptors in COS-7 and HEK293 cells. In HEK293 cells, the observed ERK1/2 activation was Ras-dependent but Rap1-independent and involved one or more Raf isoforms. Therefore, we suggest the following pathway for activation of ERK1/2 by 5-HT through 5-HT_{4(b)} and 5-HT_{7(a)} receptors: G_s-coupled 5-HT receptor → cAMP → PKA → Ras → Raf → MEK → ERK1/2. The unknown pathway from cAMP/PKA to Ras activation remains a challenge for future research.

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