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Serotonin 5-HT2a and 5-HT2c Receptors Stimulate Amyloid Precursor Protein Ectodomain Secretion*

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Alzheimer's disease amyloid consists of amyloid β-peptides (Aβ) derived from the larger precursor amyloid precursor protein (APP). Non-amyloidogenic APP processing involves regulated cleavage within the Aβ domain followed by secretion of the ectodomain (APPs). APPs secretion can be stimulated by muscarinic acetylcholine receptors coupled to phospholipases and kinases. To determine whether other receptor classes can regulate APP processing, we examined the relation between serotonin receptors and APPs secretion. Serotonin increased APPs release 3–4-fold in 3T3 cells stably overexpressing 5-HT2aR or 5-HT2cR. The increase was dose-dependent and was blocked by serotoninergic antagonists. Phorbol esters also increased APPs secretion, but neither kinase inhibitors nor down-regulation of PKC blocked the serotonin-induced increase in APPs secretion. Thus PKC is not necessary to stimulate APPs secretion. Phospholipase A_2 (PLA_2) inhibitors blocked the 5-HT2aR-mediated increase in APPs secretion, suggesting a role of PLA_2 in coupling 5-HT2aR to APP processing. In contrast, coupling of 5-HT2cR to APPs secretion involved both PKC and PLA_2. Serotonin also stimulated the release of the APLP2 ectodomain, suggesting that additional members of the APP multigene family are processed via similar regulated pathways. Inasmuch as generation of APPs precludes the formation of amyloidogenic derivatives, serotonin receptors provide a novel pharmacological target to reduce these derivatives in Alzheimer's disease.

The amyloid precursor protein (APP)[1] is an ubiquitous membrane-spanning glycoprotein (Kang *et al.*, 1987; Weidemann *et al.*, 1989) that is present at high levels in brain cells. It is the biological precursor of the amyloid β-protein (Aβ), the principal proteinaceous component of amyloid plaques in brains of Alzheimer's disease patients (for review, see Selkoe (1994)). APP or its derivatives may be involved in the pathogenesis of Alzheimer's disease because several familial forms of the disease are linked to APP mutations within or close to the β-amyloid domain. In cell culture, these mutations cause misprocessing of APP and related increases in the formation of Aβ (Cai *et al.*, 1993; Citron *et al.*, 1992, 1994) or the generation of longer than normal Aβ molecules (Suzuki *et al.*, 1994). Moreover, the overexpression of APP in transgenic mice causes formation of brain amyloid plaques (Games *et al.*, 1995) that resemble those in Alzheimer's disease.

APP is a secretory glycoprotein. The secreted APP ectodomain (APPs) has neurotrophic and neuroprotective activities in selected experimental systems. It promotes neurite outgrowth and branching in PC-12 cells (Milward *et al.*, 1992), mediates cell adhesion in cultured fibroblasts (Saitoh *et al.*, 1989), and it can protect primary neuronal cultures from excitotoxic damage (Barger *et al.*, 1995; Mattson *et al.*, 1993). Furthermore, moderate overexpression of human APP in mouse brain can increase synaptic density (Mucke *et al.* 1994), and it protects neurons *in vivo* from neurotoxic events caused by overexpression of the HIV-1 surface protein pg120 (Mucke *et al.*, 1995b) or by excitotoxic damage (Mucke *et al.*, 1995a). In selected experimental systems, APPs activates cellular signaling pathways, including $p21^{ras}$ microtubule-associated protein kinases (Greenberg *et al.*, 1994), and cGMP-mediated signaling in primary hippocampal neurons (Barger *et al.*, 1995). Secretory APP processing involves either proteolytic cleavage within the Aβ domain (Esch *et al.*, 1990; Sisodia *et al.*, 1990) or, alternatively, cleavage at both the N and C termini of Aβ followed by rapid secretion of Aβ (Haass *et al.*, 1992; Shoji *et al.*, 1992). Thus, APP can be processed by at least two alternative secretory pathways to yield either APPs and p3, a derivative of the cell-associated C terminus produced by APPs secretion (Haass *et al.*, 1993), or Aβ and a C-terminally truncated form of APPs (Seubert *et al.*, 1993). Secretory APP processing and the formation of APPs can be readily accelerated by an unusual mechanism coupled to a variety of extra- and intracellular signals. These include muscarinic acetylcholine receptors (Buxbaum *et al.*, 1992; Nitsch *et al.*, 1992; Wolf *et al.*, 1995), metabotropic glutamate receptors (Lee *et al.*, 1995), protein kinase C (PKC) (Caporaso *et al.*, 1992; Slack *et al.*, 1993), tyrosine kinases (Slack *et al.*, 1995), and arachidonic acid (Emmerling *et al.*, 1993). In rat brain tissue slices, the regulation of APP processing appears to be a function of neuronal activity: APPs secretion is stimulated by electrical depolarization in a frequency-dependent, tetrodotoxin-sensitive fashion (Nitsch *et al.*, 1993), and by muscarinic receptor agonists (Farber *et al.*, 1995). The rate of Aβ formation appears to be inversely coupled to the rate of APPs secretion: in several cell culture systems, stimulated APPs secretion was accompanied by reductions in secreted Aβ (Buxbaum *et al.*, 1993; Gabuzda *et al.*, 1993; Hung *et al.*, 1993; Wolf *et al.*, 1995), suggesting that stimulated secretory processing of APP into secreted APPs is associated with reduced formation of potentially amyloidogenic derivatives. The physi-

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[1] The abbreviations used are: APP, amyloid precursor protein; APPs, secreted APP ectodomain; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; PI, phosphatidylinositol; PMA, phorbol myristate acetate; PLA_2, phospholipase A_2.

ological relevance of regulated APPs secretion is unknown, but may be related to the functions of APPs as a paracrine neurotrophic factor or a signaling molecule that is secreted in an activity-dependent manner in response to neuronal activation by neurotransmitters.

In Alzheimer's disease brains, neurotransmission is impaired. In particular, cortical serotoninergic, glutamatergic, and peptidergic systems as well as the subcortical cholinergic projection systems are heavily damaged, indicating deafferentation of multiple cortical and hippocampal target regions. Inasmuch as neuronal activation may be involved in regulating APP processing, impaired neuronal signaling may cause alterations in APP processing pathways (for review, see Nitsch and Growdon (1994)).

In the series of experiments reported here, we examined the effects of serotoninergic receptors on APP processing using 3T3 fibroblast lines that stably overexpress 5-HT2a and 5-HT2c receptor subtypes. Wild-type 3T3 cells lack endogenous expression of serotonin receptors, and they provide the necessary cell biological background to assure appropriate signaling functions of transfected receptor subtypes (Julius *et al.*, 1988; Stam *et al.*, 1992). Furthermore, 3T3 cells process APP via regulated cleavage and secretion (Slack *et al.*, 1993). They thus provide a valid model system to study the effects of specific transfected serotonin receptor subtypes on signaling as well as on APPs secretion.

MATERIALS AND METHODS

Tissue Culture—Mouse 3T3 fibroblast lines stably overexpressing 5-HT2aR or 5-HT2cR (Julius *et al.*, 1988) were generously provided by David Julius. Transfected lines were maintained in the presence of 500 μg/ml G418. Cells were plated onto poly-D-lysine-coated dishes and grown to 75% confluence in serum-containing (10% fetal calf serum) DMEM/F-12 medium, in the absence of G418. 24 h before an experiment, the culture medium was replaced with serum-free DMEM/F-12. Functional integrity of transfected receptors was assured by measuring receptor-mediated breakdown of metabolically labeled phosphatidylinositol (PI) (Sandmann *et al.*, 1991).

Drugs—Serotonin (Sigma) was prepared freshly before each experiment and was used at 100 pM to 100 μM. The serotonin receptor antagonists ketanserin tartrate (20 μM), mianserin HCl (50 μM), and ritanserin (20 μM) were purchased from Research Biochemicals International (Natick, MA). Dexnorfenfluramine was obtained from Servier (Paris, France) and was used at 10 nM to 1 mM. Staurosporine (1 μM), chelerythrine chloride (1 μM), and melittin (2.5–5 μg/ml) were purchased from Calbiochem. Phorbol myristate acetate (Sigma) was used at 1 μM for both stimulation and 24-h down-regulation protocols. Manoalide (3.2–10 μM), 7,7-dimethyleicosadienoic acid (20–50 μM), and oleyloxyethyl phosphorylcholine (0.1–1 μM) were purchased from Biomol (Plymouth Meeting, PA). Thapsigargin was purchased from Research Biochemicals International and was used at 20 nM.

PI Turnover—For PI turnover assays, cells were labeled metabolically overnight with 1.25 μCi/dish of myo-[2-^{3}H]inositol (20.5 Ci/mmol; DuPont NEN) in inositol-deficient and serum-free DMEM/F-12 medium, washed twice with Hanks' balanced salt solution, and treated for 10 min with 10 mM lithium chloride. Drugs were added in the presence of 10 mM lithium for 5–60 min at 37 °C. To determine the amount of water-soluble inositol phosphates formed, cells were lysed with ice-cold methanol, and lipids were removed by chloroform/methanol/water (2:2:1; v:v) extraction. Water-soluble inositol phosphates were separated from free inositol by ion exchange chromatography, using AG 1-X8 columns (Bio-Rad) and 1 M ammonium formate and 0.1 M formic acid as eluent. Radioactivity was measured by liquid scintillation spectrometry.

APPs Secretion—Cells were washed twice with serum-free DMEM/F-12 medium and were incubated at 37 °C in the presence or absence of test substances freshly dissolved in DMEM/F-12. After discrete time intervals of 5–60 min, conditioned media were collected, cooled to 4 °C, and centrifuged, and supernatant fluids were desalted by gel filtration chromatography using Sephadex G-25 (Pharmacia Biotech Inc.) columns, with water as eluent. Desalted proteins were dried by vacuum centrifugation, reconstituted in water followed by 2 × Laemmli gel loading buffer, and boiled for 3 min. Cell protein was extracted with 2% SDS in Tris-buffered saline and was quantitated by the bicinchoninic acid assay (Pierce). Equal volumes of reconstituted secretory protein solutions normalized to total cell protein were separated by SDS-polyacrylamide gel electrophoresis, electroblotted onto polyvinylidene difluoride (Immobilon-P, Waters) membranes, and blocked with 5% non-fat dry milk (Carnation) in Tris-buffered saline containing 0.05% Tween 80. Membranes were probed with antibodies directed against various domains of APP and APLP2. Specifically, the monoclonal antibody 22C11, as well as the polyclonal antisera R1736, anti-C8 and D2-1 were used. Secondary antibodies were visualized on preflashed x-ray films (Kodak) by enhanced chemiluminescence (ECL, Amersham Corp.). Immunoreactive bands were quantitated by laser scanning densitometry with an LKB ultroscan densitometer set to 40-μm vertical interval size and 2.4-mm horizontal slit width. Areas under the optical density curves were expressed as arbitrary units and normalized to areas generated by immunoreactive proteins secreted under control conditions determined on the same blot. Measurements were performed in the linear range of the ECL reaction as determined from serial dilution curves of secreted proteins. Control and stimulated conditions were always handled in parallel, processed identically, and run in parallel lanes on the same blot. All experiments were done in triplicate dishes and were repeated at least three times. Statistical analysis was performed by analysis of variance, using treatments as the independent variable.

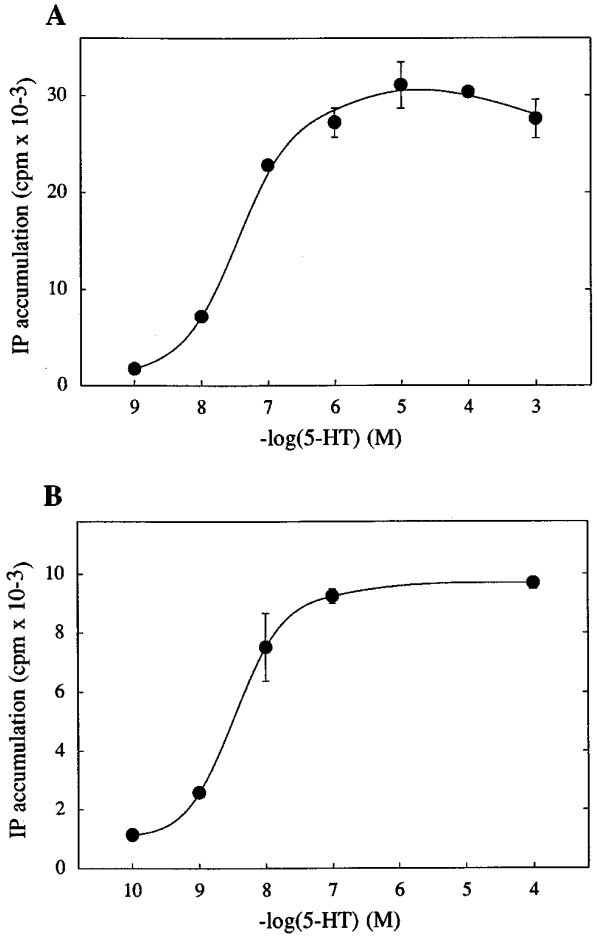

FIG. 1. **Serotonin stimulates phosphatidylinositol turnover in 3T3 fibroblasts stably expressing 5-HT2a or 5-HT2c receptors.** Cells were metabolically labeled with [^{3}H]inositol overnight, stimulated with increasing concentrations of 5-HT in the presence of 10 μM lithium, and radiolabeled inositol phosphates derived from PI breakdown were measured by scintillation counting. *A*, 3T3 cells stably expressing 5-HT2aR. *B*, 3T3 cells stably expressing 5-HT2cR. Data are from representative experiments and are means ± S.D. of triplicate culture dishes. Similar dose-response curves were obtained in four independent experiments for each cell line. *IP*, radiolabeled inositol phosphates.

RESULTS

In 3T3 fibroblasts stably transfected with cDNA expression constructs encoding either 5-HT2aR or 5-HT2cR, serotonin

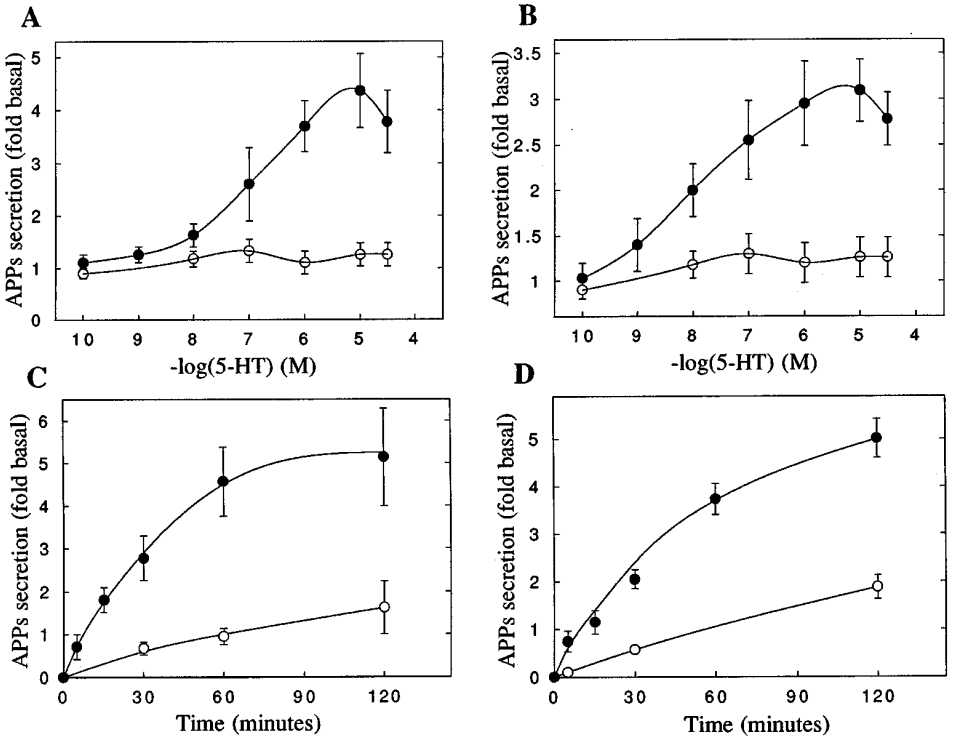

FIG. 2. **APPs secretion is increased by 5-HT receptor stimulation in 3T3 cells overexpressing 5-HT2aR or 5-HT2cR.** Cells were stimulated with 5-HT and APPs secreted into the culture medium was measured by Western blotting and densitometry. *A*, dose response of 5-HT2aR-mediated APPs secretion. *Filled symbols*, stably transfected cells; *open symbols*, untransfected parent cell line. *B*, dose response of 5-HT2cR-induced APPs secretion. *Filled symbols*, stably transfected cells; *open symbols*, untransfected parent cell line. *C*, time course of 5-HT2aR-mediated APPs secretion. *Filled symbols*, 10 μM 5-HT; *open symbols*, vehicle control. *D*, time course of 5-HT2cR-mediated APPs secretion. *Filled symbols*, 10 μM 5-HT; *open symbols*, vehicle control. Data are means ± S.E. of three independent experiments.

caused rapid and dose-dependent increases in PI turnover (Fig. 1). Serotonin increased PI turnover to 29-fold (range 7.8–53, n = 4) compared to that observed basally in cells overexpressing 5-HT2aR (Fig. 1*A*) and to 7.9-fold (range 4.1–9.9, n = 4) basal levels in cells overexpressing 5-HT2cR (Fig. 1*B*). The EC_{50} of the inositol phosphate (*IP*) accumulation in response to serotonin was 30 nM in the 5-HT2aR-expressing cells and 2 nM in the 5-HT2cR-expressing cells. Serotonin also caused APPs secretion to increase in both cell lines (Fig. 2). The maximal stimulation of APPs secretion was 4.5-fold (range 2.3–10.8, n = 9) basal levels in cells expressing 5-HT2aR and 3-fold (range 1.6–3.9) in cells expressing 5-HT2cR. The EC_{50} of 5-HT-mediated APPs secretion was 200 nM in cells expressing 5-HT2aR (Fig. 2*A*) and 120 nM in the 5-HT2cR-expressing cells (Fig. 2*B*). Time course analyses showed that the 5-HT-mediated stimulation of APPs secretion was rapid, with significant increases in both cell lines occurring as soon as 5 min following the application of 5-HT (Fig. 2, *C* and *D*). The increase in APPs secretion caused by 5-HT2aR stimulation was blocked by the serotoninergic antagonists ketanserin, mianserin, and ritanserin (Fig. 3), and the 5-HT2cR-mediated increase was blocked by mianserin (Fig. 3), indicating that the effects on APP processing in both cell lines were specifically mediated by activation of the overexpressed 5-HT2 receptor subtypes. Direct stimulation of PKC by the phorbol ester PMA mimicked the 5-HT-induced increase in APPs secretion (Figs. 4*A* and 5*A*). In both cell lines, however, the kinase inhibitors staurosporine and chelerythrine chloride failed to block the response to 5-HT (Figs. 4*A* and 5*A*). Moreover, down-regulation of PKC in these cells by chronic (24 h) pretreatment with PMA also failed to block the ability of 5-HT to stimulate APPs secretion (Figs. 4*A* and 5*A*). The PMA response, however, was completely blocked by prior chronic pretreatment with PMA (Figs. 4*A* and 5*A*). In 5-HT2aR-expressing cells, the 5-HT-mediated increase in APPs secretion

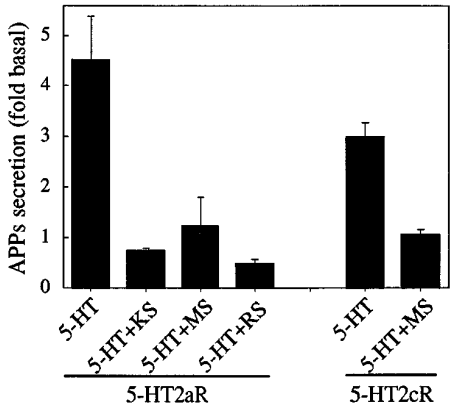

FIG. 3. **APPs secretion induced by stimulating 5-HT receptors is blocked by 5-HT receptor antagonists.** Cells were incubated with the antagonists 10 min prior to treatment with 10 μM 5-HT, and the antagonists and APPs in the culture media were quantitated by Western blotting and densitometry. *5-HT*, 10 μM serotonin; *KS*, 20 μM ketanserin; *MS*, 50 μM mianserin; *RS*, 20 μM ritanserin. Data are means ± S.E. of three independent experiments.

was effectively blocked by the PLA_2-inhibitors manoalide (*ML*), dimethyleicosadienoic acid (*DEDA*), and oleyloxyethyl phosphorylcholine (*OPC*) (Fig. 4*B*), indicating that PLA_2 may be also involved in the cellular signaling cascade that couples 5-HT2aR activation to APPs secretion. This hypothesis is supported by the finding that activation of PLA_2 by melittin mimicked the 5-HT-mediated increase in APPs secretion (Fig. 4*B*), while the calcium-releasing agent thapsigargin failed to change APPs secretion from these cells (Fig. 4*B*). Down-regulation of PKC by prior chronic preincubation with PMA (Fig. 4*C*) failed to block either the inhibition of APPs secretion by PLA_2 inhibitors or its stimulation by melittin, indicating that the signaling mediated by PLA_2 is independent of cellular PKC activity.

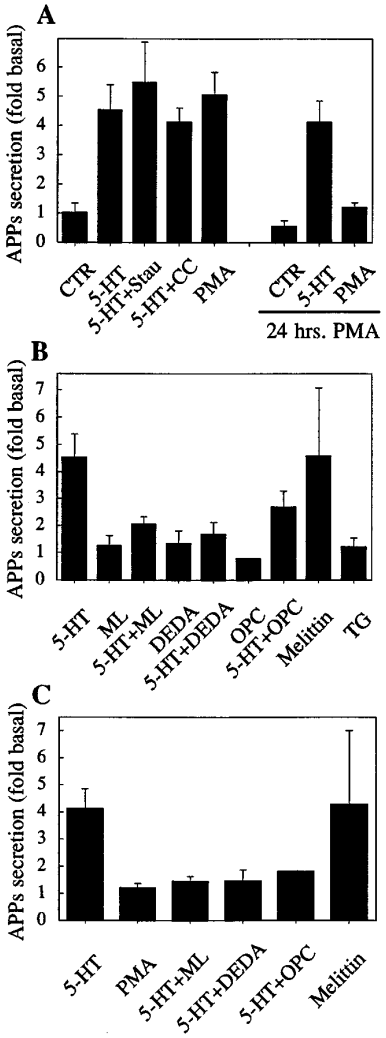

FIG. 4. **PLA_2 couples activation of 5-HT2aR receptors to increased APPs secretion.** *A*, no inhibition of 5-HT-mediated APPs secretion by the protein kinase inhibitors staurosporine (1 μM; *Stau*) and chelerythrine chloride (1 μM, *CC*). Direct activation of PKC by phorbol myristate acetate (1 μM; *PMA*) increased APPs secretion. After down-regulation of PKC by 24-h pretreatment with PMA, the response to PMA was indifferent from the vehicle control condition (*CTR*). In contrast, 5-HT stimulated APPs secretion after PMA pretreatment. *B*, PLA_2 inhibitors manoalide (3.2 μM; *ML*), dimethyleicosadienoic acid (20 μM; *DEDA*), and oleyloxyethyl phosphorylcholine (0.1 μM; *OPC*) blocked 5-HT-mediated increase in APPs secretion. No alteration of basal APPs secretion by these PLA_2 inhibitors. The PLA_2 activator melittin (2.5 μg/ml) increased APPs secretion. No effect of the intracellular calcium-releasing agent thapsigargin (*TG*) on APPs secretion. *C*, inhibition and stimulation of PLA_2 after PKC down-regulation. 5-HT-mediated APPs secretion is effectively blocked by the PLA_2 inhibitors, and melittin (2.5 μg/ml) retained its ability to increase APPs secretion. Data are means ± S.E. of three experiments.

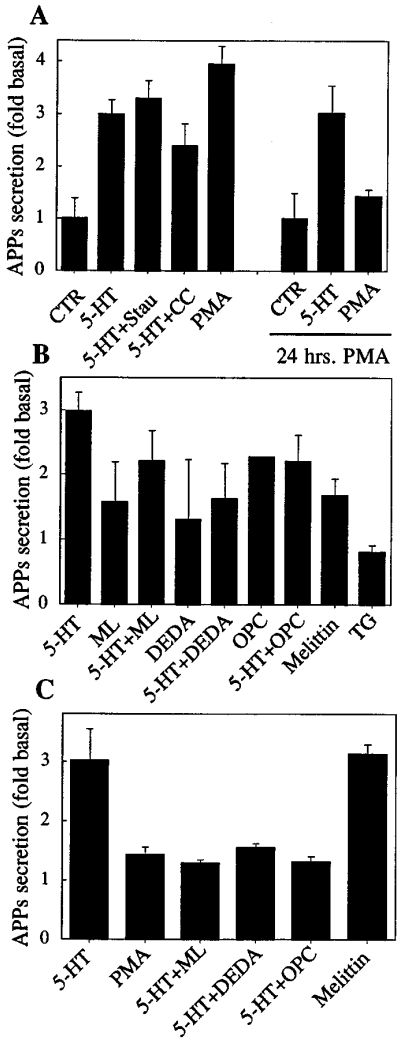

FIG. 5. **Combined action of PKC and PLA_2 in coupling 5-HT2cR receptor activation to increased APPs secretion.** *A*, no inhibition of 5-HT-mediated APPs secretion by the protein kinase inhibitors staurosporine (1 μM; *Stau*) and chelerythrine chloride (1 μM; *CC*). Direct activation of PKC by phorbol myristate acetate (*PMA*) increased APPs secretion. After down-regulation of PKC by 24-h pretreatment with PMA, the response to PMA was indifferent from the vehicle control condition (*CTR*). In contrast, 5-HT stimulated APPs secretion after PMA pretreatment. *B*, PLA_2 inhibitors manoalide (*ML*), dimethyleicosadienoic acid (*DEDA*), and oleyloxyethyl phosphorylcholine (*OPC*) failed to block 5-HT-mediated increase in APPs secretion. No alteration of basal APPs secretion by the PLA_2 activator melittin. No effect of the intracellular calcium releasing agent thapsigargin (*TG*) on APPs secretion. *C*, inhibition and stimulation of PLA_2 after PKC down-regulation. 5-HT-mediated APPs secretion is effectively blocked by all three PLA_2 inhibitors, and melittin retained its ability to increase APPs secretion. Data are means ± S.E. of three experiments.

In the 5-HT2cR-expressing cells, the PLA_2 inhibitor dimethyleicosadienoic acid only partly blocked 5-HT2cR-induced APPs secretion, and melittin was less effective in stimulating this secretion than in the 5-HT2aR-expressing cells (Fig. 5*B*). When PKC was down-regulated by prior chronic preincubation with PMA, all PLA_2 inhibitors effectively blocked the 5-HT2cR-mediated increase in APPs secretion (Fig. 5*C*), suggesting that PKC and PLA_2 are both involved in coupling of 5-HT2cR activation to APPs secretion. The serotoninergic agonist dexnorfenfluramine (*DNF*) stimulated PI breakdown in 3T3 cells expressing 5-HT2cR, indicating that it is a potent agonist of these receptors (Fig. 6*A*). DNF dose-dependently stimulated APPs secretion in the 5-HT2cR expressing cells (Fig. 6B), and this effect was inhibited by the serotonin receptor antagonists ketanserin, mianserin, and ritanserin (Fig. 6*C*). In related experiments with cells that stably overexpress 5-HT2aR, serotonin also increased PI turnover as well as the secretion of APPs, indicating that DNF is a nonselective agonist of both 5-HT2aR and 5-HT2cR (data not shown). In contrast to the responses of stably transfected cells lines, the untransfected parent cell line failed to exhibit changes in APPs secretion when exposed to serotonin (100 pM to 100 μM).

Increased APPs secretion was detected by the nonselective monoclonal antibody 22C11 (Fig. 7), as well as by the antiserum R1736 (Fig. 8), an APP-specific antiserum directed against the N-terminal 16 residues of the Aβ domain. This observation, in combination with the finding that the anti-

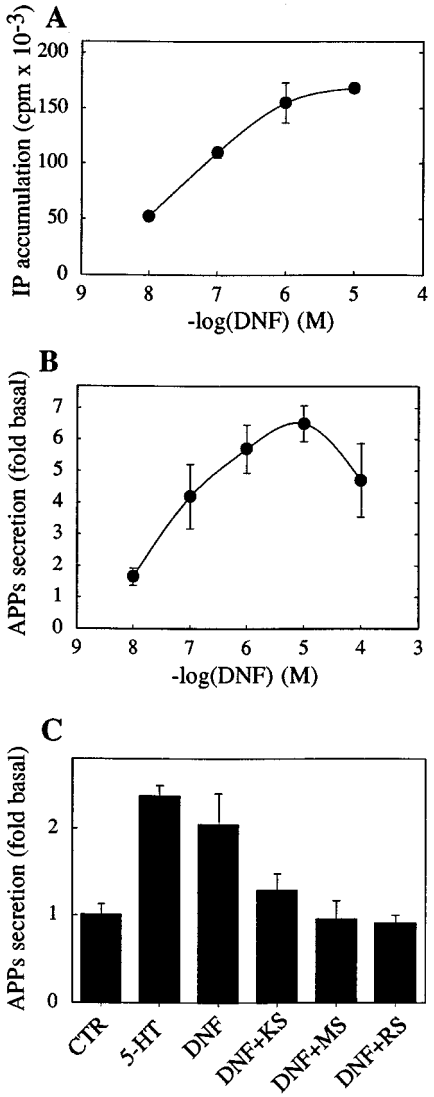

FIG. 6. **Dexnorfenfluramine increases APPs secretion.** *A*, stimulation of 5-HT2aR-coupled PI turnover by increasing concentrations of dexnorfenfluramine (*DNF*). *IP*, radiolabeled inositol phosphates. *B*, DNF increased APPs secretion in 3T3 cells expressing 5-HT2aR. *C*, DNF-induced stimulation of APPs secretion was blocked by the 5-HT receptor antagonists ketanserin (20 μM; *KS*), mianserin (50 μM, *MS*), and ritanserin (20 μM; *RS*). Data are means ± S.E. from triplicate culture dishes of representative experiments.

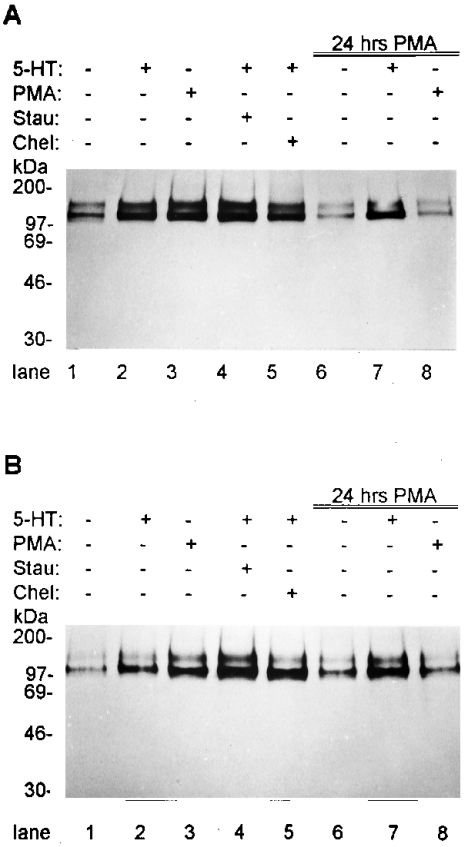

FIG. 7. **Western blot of secreted APPs with the monoclonal antibody 22C11.** *A*, 3T3 cells stably transfected with cDNA encoding 5-HT2aR. *B*, 3T3 cells stably transfected with cDNA encoding 5-HT2cR. Cell were incubated for 60 min with 10 μM serotonin (*5-HT*), 1 μM phorbol myristate acetate (*PMA*), 1 μM staurosporine (*Stau*), 1 μM chelerythrine chloride (*Chel*). *Lanes 6–8*, cells were preincubated with 1 μM PMA for 24 h prior to the experiments. Media were analyzed for APPs using the monoclonal antibody 22C11 (*lanes 1–5*).

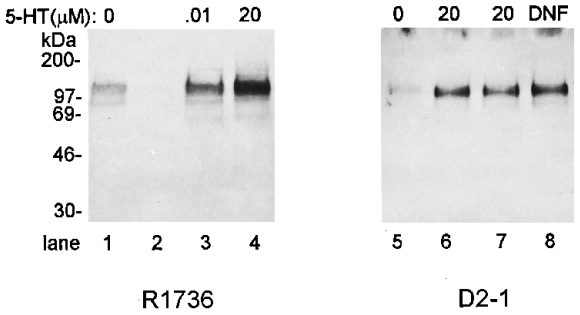

FIG. 8. **Western blots of secreted APPs and APLP2s with the specific antisera R1736 and D2-1.** APPs, secreted APPs detected by the APP-specific antiserum R1736 directed against the 16 N-terminal residues of the Aβ domain (absent in APLP2). R1736 detects increased signals in 5-HT-treated 3T3 cells (*lane 3*, 0.01 μM 5-HT; *lane 4*, 20 μM 5-HT stably transfected with 5-HT2cR as compared with vehicle-treated transfected cells (*lane 1*). APLPs, secreted APLP2s detected by the APLP2-specific antiserum D2-1 raised against baculovirus-expressed full-length APLP2. This antiserum does not cross-react with APPs. Compared with the vehicle-treated control condition (*lane 5*), D2-1 detected increased signals in 5-HT-treated (10 μM; *lanes 6* and *7*) and DNF-treated (1 μM; *lane 8*) 3T3 cells transfected with 5-HT2cR.

serum anti-C8 raised against the C terminus did not detect any protein in the culture medium (data not shown), suggests that secreted APPs was derived by conventional α-secretase processing. The anti-serum D2-1, raised against full-length APLP2 expressed in a baculovirus system (Slunt *et al.*, 1994), detected a secreted APLP2 derivative of the expected molecular mass in the conditioned culture media from cells overexpressing 5-HT2cR (Fig. 8). Both serotonin and dexnorfenfluramine substantially increased this APLP2s secretion.

DISCUSSION

The results of this study show that the rates at which APPs is secreted by cultured 3T3 cells stably overexpressing 5-HT2aR and 5-HT2cR can be accelerated by serotonin or by the serotoninergic agonist DNF. This finding, coupled with previous observations that stimulating muscarinic acetylcholine receptor subtypes m1 and m3 (Buxbaum *et al.*, 1992; Nitsch *et al.*, 1992; Wolf *et al.*, 1995) or metabotropic glutamate receptors (Lee *et al.*, 1995) can increase APPs secretion, suggests that the regulation of APP processing may be a function of various cell surface receptor types, linked to a number of common second messenger systems.

Stimulation of 3T3 cells overexpressing 5-HT2aR or 5-HT2cR caused rapid and dose-dependent increases in phosphatidylinositol breakdown, as indicated by the accumulation of labeled inositol phosphates in the presence of lithium after

metabolic labeling of phosphatidylinositol. These results confirm that both receptor subtypes are coupled to PI turnover signaling and assure that the stably transfected cell lines express functionally intact surface receptor proteins. Two additional lines of evidence demonstrated that stimulated APPs secretion was mediated by the activation of serotoninergic cell surface receptors: 5-HT failed to change APPs secretion from the untransfected parent 3T3 fibroblast line, and the increase in APPs secretion from the stably transfected cells was blocked by 5-HT receptor antagonists.

In agreement with previous reports (Caporaso *et al.,* 1992; Slack *et al.,* 1993), direct activation of PKC with the phorbol ester PMA increased APPs secretion in the cell lines used in this study. In contrast to our previous observations with 293 cells overexpressing m1 and m3 acetylcholine receptors (Nitsch *et al.,* 1992), however, inhibition of PKC by the kinase inhibitors staurosporine or chelerythrine chloride failed to block 5-HT-mediated increases in APPs secretion from 3T3 fibroblasts. Moreover, down-regulation of PKC by prior chronic pretreatment with PMA also failed to block the 5-HT-induced increase in APPs secretion (Fig. 4*A*). These data imply that activation of PKC can be sufficient to increase APPs secretion, but that this activation is not necessary for the 5-HT2R-mediated increase. The concentrations of 5-HT necessary to elicit maximal acceleration of PI breakdown, as well as the EC_{50} of this effect, were 1 to 2 orders of magnitude lower than these for stimulated APPs secretion. This is compatible with the possibility that APP processing is also coupled to other cellular signaling pathways besides PI turnover and PKC activation. To test whether PLA_2 is involved in 5-HT-mediated APPs secretion, we examined the ability of drugs that inhibit this enzyme, and we also used melittin, a PLA_2-activating peptide. Stimulation of 5-HT2aR-expressing cells with 5-HT in the presence of the PLA_2 inhibitors manoalide, dimethyleicosadienoic acid, or oleyloxyethyl phosphorylcholine failed to affect basal APPs secretion, but all three drugs inhibited 5-HT-induced secretion. These results demonstrate that 5-HT2aR activation can accelerate APPs secretion by coupling to PLA_2. A role of PLA_2 in the regulation of APPs processing was also suggested by the finding that melittin accelerated APPs secretion. Moreover, this effect was not blocked by down-regulation of PKC by chronic exposure to PMA, and manoalide, dimethyleicosadienoic acid, or oleyloxyethyl phosphorylcholine blocked 5-HT-induced increases in APPs secretion after down-regulation of PKC. Hence, 5-HT2aR-coupled and PLA_2-mediated acceleration of APPs secretion can occur independently of PKC. These data are consistent with the previous observation that PLA_2 can partially mediate the increase in APPs secretion induced by muscarinic stimulation (Emmerling *et al.,* 1993).

Cellular signaling mechanisms that couple 5-HT2cR to APP processing were more complex than in the cells overexpressing 5-HT2aR. As with 5-HT2aR activation, cells expressing 5-HT2cR responded with increased APPs secretion to 5-HT after down-regulation of PKC, and the kinase inhibitors staurosporine and chelerythrine chloride failed to inhibit this increase. In contrast to the findings in 5-HT2aR cells, however, the PLA_2 inhibitors failed to block consistently the 5-HT-mediated increase in APPs secretion in 5-HT2cR cells. The combination of PKC down-regulation and PLA_2 inhibition did effectively inhibit the ability of 5-HT to stimulate APPs secretion. It is therefore possible that the coupling of 5-HT2cR to APPs secretion requires both PKC and PLA_2 activities. Treatment of the 5-HT2R-expressing cells with thapsigargin, which discharges Ca^{2+} from internal stores by inhibiting the Ca^{2+}-ATPase of the endoplasmic reticulum membrane failed to affect APPs secretion in the 3T3 cells used. Perhaps the previously reported effects of Ca^{2+} on APPs secretion (Buxbaum *et al.,* 1994) are cell type-specific.

APP is a member of the multigene family of APP-like proteins (APLP). APLP2 is homologous to APP in the extreme C-terminal region, and has various homologous regions in the ectodomain (Slunt *et al.,* 1994; von der Kammer *et al.,* 1994; Wasco *et al.,* 1992, 1993). However the Aβ domain is not preserved; thus no amyloidogenic derivatives can be generated from APLP2. Because of its similarities to APP, APLP2 is detected by nonselective antibodies (Slunt *et al.,* 1994), including the monoclonal antibody 22C11 that was raised initially against an APP fusion protein (Weidemann *et al.,* 1989). To differentiate between APPs and APLPs, we used the antiserum R1736 (Haass *et al.,* 1992) directed against the 16 N-terminal residues of the Aβ domain. R1736 detected the same receptor-mediated increase in secreted APPs as 22C11 (Fig. 8). The APLP2-specific antiserum D2-1 (Slunt *et al.,* 1994) detected secreted APLP2 derivatives (APLP2s) with the expected molecular masses in the cell culture supernatants obtained from 3T3 cells overexpressing 5-HT2aR or 5-HT2cR. Stimulation of these cell lines with 5-HT substantially increased APLP2 s secretion (Fig. 8). These data suggest that APP and APLP2 are processed by similar receptor-regulated proteolytic pathways, and they imply the possibility that APLP2 may compete with APP for enzymes involved in signal transduction and proteolytic processing. Levels of APLP2 may thus influence the metabolism of APP. The cellular mechanism of receptor-regulated APPs and APLP2s secretion is unclear. As in previously reported experiments, 5-HT2R-mediated increases in APPs secretion occurred in the absence protein synthesis confirming that pre-existing protein is processed in response to receptor activation. It is possible that secretory vesicle formation, trafficking, or proteolytic cleavage events are accelerated by surface receptor-coupled signaling. The first possibility is underscored by the recent finding that PKC stimulates vesicle budding from the trans-Golgi network and thus accelerates the formation of APP-containing secretory vesicles in a reconstituted cell-free system (Xu *et al.,* 1995).

The physiological relevance of regulated APPs secretion is unclear. We speculate that having APP processing under neurotransmitter control may allow neuronal activity to control the formation of a secretory derivative (Farber *et al.,* 1995; Nitsch *et al.,* 1993) with possible paracrine neurotrophic and neuroprotective activities. Regulated cleavage of a membrane precursor followed by secretion of the ectodomain has been described for a variety of transmembrane proteins, including transforming growth factor-α (Bosenberg *et al.,* 1992; Pandiella *et al.,* 1991), and the tumor necrosis factor receptor (Brakebusch *et al.,* 1992), and it was proposed that regulated secretion is involved in switching transforming growth factor-α's activity from that of a juxtacrine to a paracrine growth factor (Pandiella and Massague, 1991). Growth factor-like activities of APPs have been observed in several cell culture models. In particular, a neurotrophin-like stimulation of neurite outgrowth and branching was observed in PC-12 cells (Milward *et al.,* 1992). Conversely, antisense constructs directed against APP transcripts inhibit neurite outgrowth in primary neurons (Allinquant *et al.,* 1995). A biologically active domain that may promote trophic activities of APP *in vivo* was mapped to the N terminus of APPs (Roch *et al.,* 1994). APPs also protects primary neurons from glutamate-induced excitotoxic damage (Mattson *et al.,* 1993), presumably by suppressing potentially toxic increases in intracellular calcium (Barger *et al.,* 1995). Thus, it is possible that secreted APPs has trophic functions in brain that are unrelated to APP's role as an amyloid precursor.

Regulated APPs secretion can be mediated by cholinergic

(Buxbaum *et al.*, 1992; Nitsch *et al.*, 1992; Wolf *et al.*, 1995) and glutamatergic (Lee *et al.*, 1995) agonists, and electrical activity of brain cells activated APPs secretion from freshly prepared tissue slices. Hence, receptor-coupled APP processing may normally occur throughout the brain at muscarinic, glutamatergic, and, now, serotoninergic synapses. In most (Buxbaum *et al.*, 1993; Gabuzda *et al.*, 1993; Hung *et al.*, 1993; Wolf *et al.*, 1995), but not all (Dyrks *et al.*, 1994) cell types, receptor-mediated activation of APPs secretion is associated with decreased generation of Aβ. We were unable to measure Aβ secreted from the 3T3 fibroblasts within the short time intervals used for receptor stimulation in this study. Because cultured fibroblasts secrete very little amounts of Aβ, time periods of more than 24 h are necessary to detect measurable levels with current immunoadsorbent assays. Thus it remains to be investigated whether serotonin receptor-induced stimulation of APPs secretion is associated with changes in the rate of Aβ secretion.

In Alzheimer's disease brain, amyloid deposits are present throughout the brain cortex, and they are not co-localized with any specific neurotransmitter system. Similarly, many neurotransmitter systems, including the cholinergic, serotoninergic, glutamatergic, and peptidergic systems, are heavily damaged in Alzheimer's disease brains, and this damage is associated with significant losses in cortical synapses (Terry *et al.*, 1991). Inasmuch as these pathological alterations and the resulting deafferentation of target cells are associated with amyloidogenic APP processing, they may be involved in promoting the amyloid formation in Alzheimer's disease brain.

Identifying cell surface receptors, such as 5-HT2aR and 5-HT2cR, whose stimulation increases APPs secretion, could constitute a useful novel pharmacological strategy for manipulating of APP processing in brain, for promoting the potential functions of APPs as a paracrine neurotrophic/neuroprotective factor, and for concomitantly reducing the formation of amyloidogenic derivatives. A possible candidate compound may be dexfenfluramine, a widely used anti-obesity drug which is metabolized to dexnorfenfluramine *in vivo*. Brain levels of dexnorfenfluramine in subjects taking therapeutic doses of dexfenfluramine (30 mg/day) are probably in the order of 1–3 μM.[2] In our transfected cell lines, this concentration of dexnorfenfluramine was sufficient to promote both APPs secretion and PI turnover (Fig. 6). Clinical studies with highly selective receptor agonists are needed to determine whether this approach can modify the clinical course of Alzheimer's disease.

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