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Adenosine Receptor, Protein Kinase G, and p38 Mitogen-Activated Protein Kinase-Dependent Up-Regulation of Serotonin Transporters Involves Both Transporter Trafficking and Activation

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ABSTRACT

Serotonin (5-hydroxytryptamine; 5-HT) transporters (SERTs) are critical determinants of synaptic 5-HT inactivation and the targets for multiple drugs used to treat psychiatric disorders. In support of prior studies, we found that short-term (5–30 min) application of the adenosine receptor (AR) agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) induces an increase in 5-HT uptake V_{max} in rat basophilic leukemia 2H3 cells that is enhanced by pretreatment with the cGMP phosphodiesterase inhibitor sildenafil. NECA stimulation is blocked by the A_3 AR antagonist 3-ethyl-5-benzyl-2-methyl-phenylethynyl-6-phenyl-1,4(±)dihydropyridine-3,5-dicarboxylate (MRS1191), by the phospholipase C inhibitor 1-(6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl] amino]hexyl)-1*H*-pyrrole-2,5-dione (U73122), by the intracellular Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester, and by the guanyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one. Hydroxylamine, a nitric-oxide donor, and 8-bromo-cGMP, a membrane-permeant analog of cGMP, mimic the effects of NECA on 5-HT uptake, whereas the protein kinase G (PKG) inhibitor *N*-[2-(methylamino)ethy]-5-isoquinoline-sulfonamide (H8) blocks NECA, hydroxylamine, and 8-bromo-cGMP effects. NECA stimulation activates p38 mitogen-activated protein kinase (MAPK), whereas p38 MAPK inhibitors block NECA stimulation of SERT activity, as does the protein phosphatase 2A (PP2A) inhibitor calyculin A. 5-HT–displaceable [^{125}I]3β-(4-iodophenyl)-tropane-2β-carboxylic acid methylester tartrate (RTI-55) whole-cell binding is increased by NECA or sildenafil, and both surface binding and cell surface SERT protein are elevated after NECA or sildenafil stimulation of AR/SERT-cotransfected Chinese hamster ovary cells. Whereas p38 MAPK inhibition blocks NECA stimulation of 5-HT activity, it fails to blunt stimulation of SERT surface density. Moreover, inactivation of existing surface SERTs fails to eliminate NECA stimulation of SERT. Together, these results reveal two PKG-dependent pathways supporting rapid SERT regulation by A_3 ARs, one leading to enhanced SERT surface trafficking, and a separate, p38 MAPK-dependent process augmenting SERT intrinsic activity.

Serotonin (5-hydroxytryptamine; 5-HT), a neurotransmitter of the central and peripheral nervous systems, modulates a wide array of behaviors, from sleep and aggression to mood and appetite (Nestler et al., 2001). In turn, many neuropsychiatric conditions including depression, anxiety, and obsessive-compulsive disorder may feature disrupted serotonergic signaling. Inactivation of synaptic 5-HT is established by the

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine, serotonin; SERT, serotonin transporter; AR, adenosine receptor; PKG, protein kinase G; PDE, phosphodiesterase; PP2A, protein phosphatase 2A; MAPK, mitogen-activated protein kinase; PLC, phospholipase C; NECA, 5′-*N*-ethylcarboxamidoadenosine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; *R*-PIA, (*R*)-N^6-phenylisopropyladenosine; IB-MECA, N^6-(3-iodobenzyl)-*N*-methyl-5′carbamoyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; MRS1191, 3-ethyl-5-benzyl-2-methyl-phenylethynyl-6-phenyl-1,4(±)dihydropyridine-3,5-dicarboxylate; RTI-55, 3β-(4-iodophenyl)-tropane-2β-carboxylic acid methylester tartrate; RBL-2H3, rat basophilic leukemia 2H3 cell line, MTSET, 2-(trimethylammonium) ethyl methanethiosulfonate; PBS/CM, phosphate-buffered solution containing 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$; RIPA, radioimmunoprecipitation assay; CHO, Chinese hamster ovary; KRH, Krebs-Ringer-HEPES; NOS, nitric-oxide synthase; H8, *N*-[2-(methylamino)ethy]-5-isoquinoline-sulfonamide; [^{3}H]5-HT, 5-hydroxy[^{3}H]tryptamine trifluoroacetate; NET, norepinephrine transporter; U73122, 1-(6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl] amino]hexyl)-1*H*-pyrrole-2,5-dione; UK14304, 5-bromo-*N*-[4,5-dihydro-1*H*-imidazol-2-yl]-6-quinoxalinamine; LY83583, 6-anilino-5,8-quinolinedione; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1*H*-imidazol; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole; SCH58261, 5-amino-7-(phenylethyl)-2-(1-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-*c*]pyrimidime; PD98059, 2′-amino-3′-methoxyflavone; β-PMA, β-phorbol 12-myristate 13-acetate; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester.

5-HT transporter (SERT); consequently, selective 5-HT reuptake inhibitors have a prominent place in clinical psychopharmacology (Barker and Blakely, 1995). Genetic and epigenetic processes modulate SERT activity (Lesch et al., 1996; Blakely and Bauman, 2000), and altered SERT expression or regulation influence risk for disease states (Caspi et al., 2003; Ozaki et al., 2003). SERT knockout mice display altered presynaptic 5-HT homeostasis, modified 5-HT receptor sensitivities, stress-dependent behavioral modulation, and altered responses to psychostimulants (Holmes et al., 2003). Together, these studies reveal critical control mechanisms that establish appropriate levels of SERT expression, target the protein at appropriate densities to the plasma lemma, and sustain appropriate rates of transport (Blakely et al., 1997, 1998; Hoffman et al., 1998). It is clear that detailed knowledge of SERT regulation could offer new opportunities for therapeutic modulation of serotonergic signaling.

Studies with in vitro models manipulating second messengers or second messenger-linked kinases have revealed multiple pathways that support acute SERT regulation. Experiments with synaptosomes and cell lines demonstrate that SERT activity decreases after depletion of intracellular Ca^{2+} (Nishio et al., 1995; Turetta et al., 2002; Ansah et al., 2003), treatment with calmodulin inhibitors (Jayanthi et al., 1994), and phorbol esters (Qian et al., 1997; Ramamoorthy et al., 1998). In human embryonic kidney cells, protein kinase C activation and PP2A inhibition trigger transporter phosphorylation, events that are correlated with a loss of SERT surface density (Qian et al., 1997; Ramamoorthy et al., 1998; Ramamoorthy and Blakely, 1999). Protein kinase A and protein kinase G (PKG) activation also triggers SERT phosphorylation (Ramamoorthy et al., 1998), although activity changes were not observed, possibly reflecting the heterologous context of SERT expression. Phorbol esters trigger dissociation of PP2A catalytic subunits from SERT complexes (Bauman et al., 2000), revealing a dynamic association with signaling proteins. The *t*-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor protein syntaxin 1A has recently been found to associate with SERT (Quick, 2002). Together, these studies reveal basal and activity-triggered pathways supporting acute SERT regulation.

Important evidence of physiologically relevant SERT regulation arises from observations of receptor-linked modulation of SERT activity in native preparations. In neurons, 5-HT_1 class autoreceptors modify hippocampal 5-HT clearance in vivo (Daws et al., 2000). Likewise, we documented a rapid down-regulation of SERT in synaptosomes and in brain after application of the $\alpha 2$ adrenergic agonist UK14304 (Ansah et al., 2003). Miller and Hoffman (1994) first provided evidence of adenosine receptor (AR) regulation of SERT using a rat basophilic leukemia cell line (RBL-2H3). In these studies, the nonselective AR agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) triggered a cGMP-linked increase in 5-HT transport, with no change in whole-cell antagonist binding. It is interesting that Launay and coworkers (1994) described histamine-induced increases in platelet SERT activity, also linked to cGMP as well as nitric-oxide synthase (NOS) activation. To gain additional insights into receptor-modulated SERT function, we sought to specify further AR and PKG-dependent SERT modulation, focusing on the analysis of signaling pathways involved and the question of whether receptor stimulation alters SERT surface density versus modulation of transporter catalytic function.

In this report, we provide evidence to support the involvement of A_3 AR in NECA up-regulated SERT activity in RBL-2H3 cells, stimulation supported by activation of phospholipase C (PLC), cytosolic Ca^{2+}, NOS, guanyl cyclase, and PKG. Furthermore, we provide evidence that NECA stimulation engages and requires the activity of p38 MAPK. We find that NECA stimulation of RBL-2H3 cells is accompanied by increases in [^{125}I]RTI-55 surface-labeled SERT proteins. Covalent inactivation of surface SERT fails to blunt AR and p38 MAPK-dependent increases in SERT activity, consistent with stimulation that arises from enhanced surface trafficking of intracellular SERT. Using Chinese hamster ovary (CHO) cells cotransfected with AR and SERT cDNAs, we demonstrate that essential elements of this pathway can be reconstituted in heterologous cells. The failure of p38 MAPK inhibition, despite blocking A_3 AR stimulation of 5-HT uptake, to block elevations in SERT surface density in either model was striking. These findings document for the first time a two-step PKG-dependent process supporting G-protein coupled receptor stimulation of biogenic amine transporters, one involving changes in transporter trafficking and a second involving transporter catalytic activation.

Materials and Methods

Reagents and Constructs. NECA, hydroxylamine, 8-bromo-cGMP, 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), H8, LY83583, U73122, (*R*)-*N*^{6}-phenylisopropyladenosine (*R*-PIA), *N*^{6}-(3-iodobenzyl)-*N*-methyl-5′carbamoyladenosine (IB-MECA), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), and MRS1191 were obtained from Sigma Chemical (St. Louis, MO); BAPTA-AM was purchased from Calbiochem (San Diego, CA); Calyculin A, SB203580, and SB202190 were obtained from Alexis Biochemicals (San Diego, CA). 2-(Trimethylammonium)ethyl methanethiosulfonate (MTSET) was obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). Adenosine receptor cDNAs (hA1R/PCM, hA2AR/PCM, hA2BR/PCM, and hA3R/PCM) have been described previously (Klotz et al., 1998). Sildenafil was purified and kindly provided by Dr. Jackie Corbin (Corbin et al., 2003) at Vanderbilt University (Nashville, TN). 5-Hydroxy[^{3}H]tryptamine trifluoroacetate ([^{3}H]5-HT, 107 Ci/mmol) was purchased from Amersham Biosciences Inc. (Piscataway, NJ); [^{125}I]RTI-55 (3β-(4-iodophenyl)-tropane-2β-carboxylic acid methylester tartrate, 2200 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Trypsin-EDTA, glutamine, and ampicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA); modified Eagle's medium and Dulbecco's modified Eagle's medium were derived from Invitrogen reagents and prepared in the Vanderbilt Media Core. Human SERT-specific monoclonal antibodies were obtained from MAb Technologies (Atlanta, GA). Streptavidin beads and EZ-link NHS-sulfo-*S*-*S*-biotin were purchased from Pierce (Rockford, IL).

Cell Culture and Transfection. RBL-2H3 cells were maintained in modified Eagle's medium containing 15% fetal bovine serum, 1% L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. CHO cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Transfections were performed using Fugene 6 reagent (Roche Applied Science, Indianapolis, IN). SERT and/or AR cDNAs were preincubated with the reagent at ambient temperature for 30 min before adding to plated cells. In most cases, 1 μg of SERT cDNA was added to each well of a six-well plate seeded with 500,000 cells, and 0.2 μg/well was used for 24-well plates seeded at

10,000 cells/well. Amounts of AR cDNAs cotransfected with SERT were titrated to ensure sufficient expression for agonist-response profiling without altering SERT expression (0.2 μg/well for six-well plate or 0.04 μg/well for 24-well plate). Cells were cultured for 24 to 48 h after transfection before assay.

5-HT Transport Assays. Assays measuring transport of [^{3}H]5-HT were performed as described previously (Miller and Hoffman, 1994; Ramamoorthy et al., 1998). In brief, medium from RBL-2H3 or transfected CHO cells was removed by aspiration. Cells were washed once with Krebs-Ringer-HEPES (KRH) buffer containing 130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.8 g/L glucose, and 10 mM HEPES, pH 7.4. Cells were then incubated in triplicate at 37°C in KRH buffer (0.2 ml/well) containing 100 μM pargyline, 100 μM ascorbic acid, and 1.0 mM Tropolone (Sigma) with or without modifiers. After 10-min incubation with [^{3}H]5-HT (20 nM for CHO cells and 100 nM for RBL-2H3 cells) at 37°C, the buffer was aspirated and the cells were washed three times with ice-cold KRH buffer. Cells were solubilized with 0.5 ml Microscint 20 (PerkinElmer Life and Analytical Sciences), and [^{3}H]5-HT accumulation was quantified using a TopCount plate scintillation counter (PerkinElmer Life and Analytical Sciences). Specific 5-HT uptake was determined by subtracting the amount of [^{3}H]5-HT accumulated in the presence of 10 μM paroxetine. For studies seeking to inactivate surface SERTs in RBL-2H3 cells, we treated cells with the membrane-impermeant, cysteine-specific alkylating reagent MTSET (Chen et al., 1997). Cells were treated either with vehicle or with MTSET (10 mM) for 10 min at room temperature before washing and measurement of NECA-stimulated 5-HT transport as described above. All experiments were repeated at least three times. Statistical analyses, comparing baseline and compound-modified uptake, were performed using one- and two-way analysis of variance with subsequent planned comparisons (Dunnett, Bonferroni), as well as *t* tests as noted in the figure legends.

[^{125}I]RTI-55 Binding Assays. For assessment of SERT surface density, we used the high-affinity cocaine analog [^{125}I]RTI-55 to label cells and used lipophilic (5-HT) or hydrophobic (paroxetine) competitors to define surface and total specific binding, respectively. After drug treatment, binding assays were performed on ice to limit 5-HT entry into cells and limit further exocytosis/endocytosis of SERT proteins. KRH solution containing drugs was removed by aspiration and washed twice with ice-cold phosphate-buffered solution containing $CaCl_2$ (0.1 mM) and $MgCl_2$ (1.0 mM) (PBS/CM). For each condition, the cells were preincubated on ice for 5 min with 1) 1× PBS/CM for total binding; 2) 1× PBS/CM containing 100 μM 5-HT to define specific surface binding; and 3) 1× PBS/CM containing 1 μM paroxetine to define specific total binding. Cells were further incubated on ice with varying concentrations of [^{125}I]RTI-55 (final concentration = 5 nM for single-point assays) for 45 min. Binding was terminated by two rapid washes with ice-cold PBS/CM. Cells were solubilized with 1% SDS, and the amount of [^{125}I]RTI-55 bound was quantified on a Gamma 4000 counter (Beckman Coulter, Fullerton, CA).

Biotinylation and Immunoblots. To achieve analysis of changes in cell-surface SERT protein, biotinylation and immunoblots were performed after drug treatment of SERT-transfected CHO cells. Cells were seeded and transfected with AR and SERT cDNAs in six-well plates, cultured for 48 h, and treated with drugs as described above. After drug treatment, all procedures were performed on ice. Cells were washed twice with ice-cold PBS/CM and incubated with 1 ml/well EZ-link NHS-sulfo-*S*-S-biotin (1 mg/ml in PBS/CM; Pierce) for 30 min. The biotinylation reagent was quenched by two washes and 10-min incubation with 100 mM glycine in PBS/CM, followed by another two washes with PBS/CM. The cells were then lysed in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and 0.1 Na-deoxycholic acid) containing protease inhibitors (1 μM pepstatin A, 250 μM phenylmethylsulfonyl fluozide, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) for 30 min at 4°C with constant shaking. Lysates were centrifuged at 20,000*g* for 30 min at 4°C, and supernatants were incubated with streptavidin bead (30 μl beads/cell lysates from one well; Pierce) for 45 min at room temperature. Beads were washed three times with RIPA buffer, and bound proteins were eluted with 30 μl of Laemmli buffer (62.5 mM Tris, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.01% bromphenol blue) for 1 h at ambient temperature. The samples were centrifuged for 10 min and protein-analyzed by SDS-polyacrylamide gel electrophoresis (10%) before being transferred overnight at 150 mA to polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). Blotted membranes were subsequently blocked with 5% nonfat dry milk in phosphate-buffered saline with 0.1% Triton X-100 and probed with SERT monoclonal antibody (1:1000; MAb Technologies). Bound antibody was detected with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:10,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA), and horseradish peroxidase signals developed with ECL Plus solution (Amersham Biosciences). Activation of p38 MAPK was determined as reported previously (Sweeney et al., 1999; Apparsundaram et al., 2001). In brief, RBL-2H3 cells were treated with NECA for 5 min in the presence or absence of SB203580. After treatment, cells were lysed with RIPA buffer containing protease inhibitors and cell lysates were centrifuged at 20,000*g* for 30 min. Antiphosphotyrosine antibody (2 μg) was added to each supernatant and incubated overnight at 4°C. Protein A Sepharose beads was then added (3 mg/sample) and rotated for 1 h at room temperature. The beads were washed three times with RIPA buffer, and bound proteins were eluted with 50 μl of Laemmli buffer, separated by SDS-polyacrylamide gel electrophoresis, transferred and immunoblotted with p38 MAPK polycloned antibody (1:1000), and detected by ECL Plus solution. To estimate the relative abundance of proteins in total and surface immunoblots, films were scanned on an Agfa Duoscan T1200 (Agfa Gevaert, Leverkusen, Germany), and the captured images were analyzed in Adobe Photoshop (Adobe Systems, Mountain View, CA) and quantified with NIH Image software (http://rsb.info.nih.gov/nih-image/), taking care through multiple exposures to work in the linear range of the film.

Results

Activation of ARs in RBL-2H3 Cells Induces an Increase in 5-HT Uptake that Is Enhanced by Sildenafil. NECA, a nonselective AR agonist (K_i values for A_1, A_{2A}, and A_3 = 6.3, 10, and 113 nM, respectively) (Feoktistov and Biaggioni, 1997), was used to activate ARs in RBL-2H3 cells (Miller and Hoffman, 1994). We detected a dose- and time-dependent, NECA-triggered increase in 5-HT uptake (Fig. 1, A and B). In the absence of other agents, NECA stimulation of 5-HT transport was confined to a narrow concentration range (Fig. 1A). Our current report focuses on the effects of NECA at 1 μM, at which 5-HT transport stimulation was consistently observed. We examined NECA concentrations greater than 100 μM but found that shape changes and cell lifting prevented interpretations of effects at higher concentrations. The effects of NECA at 1 μM were rapid, reaching a maximum at 10 min. Consistent with prior studies (Miller and Hoffman, 1994), kinetic analyses revealed that NECA effects are supported by a change in 5-HT V_{max} with no significant change in K_m (Fig. 1C). Because NECA has been shown to elevate cGMP in RBL-2H3 cells (Miller and Hoffman, 1994), we also tested whether blockade of cGMP hydrolysis would further augment the stimulation of SERT by NECA. Indeed, we found that sildenafil (Viagra; Pfizer Inc., New York, NY), a specific inhibitor of the cGMP-specific PDE5, increased 5-HT uptake in RBL-2H3 cells (Fig. 1C) in a dose-dependent manner (data not shown), supported spe-

cifically by a V_{max} increase, and further elevated the NECA-induced increase in 5-HT transport V_{max}, achieving a 40 to 50% elevation in transport with 1 μM NECA plus 4 μM sildenafil.

A_3 but Not A_1 or A_{2A} ARs Mediate the NECA-Induced Stimulation of 5-HT Uptake in RBL-2H3 Cells. RBL-2H3 cells express predominantly A_3 AR with no evidence for the A_1 subtype (Feoktistov and Biaggioni, 1997). The availability of selective antagonists for different ARs enabled us to explore further the specific receptor subtypes involved in mediating NECA's effects on 5-HT transport. The concentrations for agents in these studies were chosen based on previously reported studies of AR antagonists (Feoktistov and Biaggioni, 1997). Full inhibition of the NECA stimulation was achieved with MRS1191 (1 μM), a specific A_3 antagonist (K_i = 31.4 nM) (Feoktistov and Biaggioni, 1997) (Fig. 2). This agent did not influence basal 5-HT transport. As expected, neither the A_1 antagonist DPCPX (1 μM) nor the A_{2A} antagonist SCH58261 (100 nM) affects NECA-stimulated 5-HT uptake.

PLC and Intracellular Ca^{2+} Are Required for AR-Stimulated 5-HT Uptake: Upstream of cGMP Production and PKG Activation. ARs couple to inhibitory Gi/o proteins (A_1 and A_3 receptors), stimulatory Gs proteins (A_{2A} and A_{2B} receptors), as well as Gq protein-linked PLC (A_1, A_{2B}, and A_3 receptors) (Feoktistov and Biaggioni, 1997; Fredholm et al., 2000). In keeping with a prominent role of A_3 receptors in the NECA stimulation of SERT in RBL-2H3 cells, we found that at a concentration that does not affect basal 5-HT uptake, the PLC inhibitor U73122 (10 μM) blocked NECA stimulation of SERT (Fig. 3A). PLC activation results in mobilization of intracellular Ca^{2+} stores in mast cells (Feoktistov and Biaggioni, 1997). In support of an involvement of this pathway, we found that the NECA stimulation of 5-HT uptake occurs in the absence of extracellular Ca^{2+}, whereas preincubation of cells with BAPTA-AM abolished NECA-stimulated 5-HT uptake (Fig. 3B). That the requirement for cytosolic Ca^{2+} lies upstream of cGMP production was established in observations that the same treatments with BAPTA-AM failed to impact sildenafil, hydroxylamine, or 8-bromo-cGMP stimulated transport activity (Fig. 3B). Restoration of extracellular Ca^{2+} (2.2 mM) to the medium (Fig. 3C) eliminated the BAPTA-AM suppression of NECA stimulation, indicating that extracellular Ca^{2+} influx can sustain NECA regulation of SERT and that cytosolic Ca^{2+} elevations may involve both release from PLC-linked cytosolic stores and plasma membrane Ca^{2+} influx. It is noteworthy that the sustained effect of the NO donor hydroxylamine under conditions of Ca^{2+} chelation indicates that the

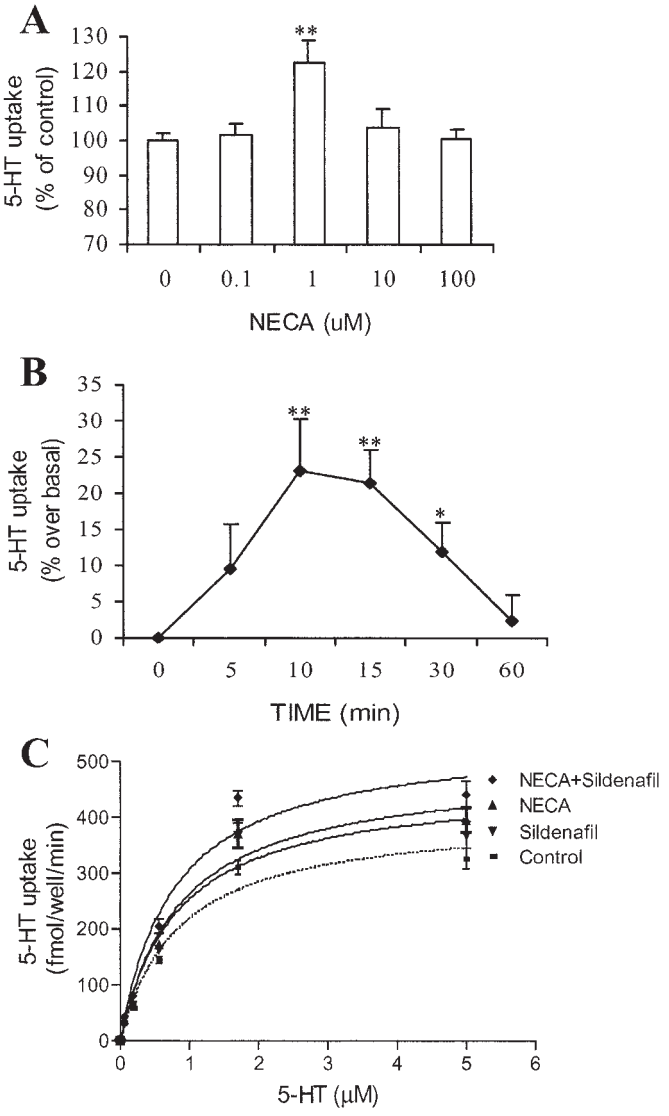

Fig. 1. Effect of NECA and/or sildenafil on 5-HT uptake in RBL-2H3 cells. A, cells (2.5×10^4/well) were treated with NECA at the indicated concentrations for 10 min before transport assay. B, influence of pretreatment duration on NECA (1 μM) stimulation of 5-HT uptake. C, kinetic analysis of NECA/sildenafil stimulation of 5-HT uptake. Cells (2.5×10^4/well) were treated individually or jointly with NECA (1 μM) or sildenafil (4 μM) or with vehicle for 10 min before transport assays. Kinetic values (V_{max}:K_m) obtained were the following: control, 405 ± 24 fmol/well/min:0.84 ± 0.15 μM; NECA treatment, 489 ± 30** fmol/well/min:0.86 ± 0.16 μM; sildenafil, 462 ± 33* fmol/well/min:0.82 ± 0.18 μM; and NECA ± sildenafil, 545 ± 34** fmol/well/min:0.77 ± 0.15 μM. Values are expressed as the mean of at least three experiments ± S.E.M. *, $p < 0.05$; **, $p < 0.01$ versus vehicle controls for both figures (A, B) and values in legend (C).

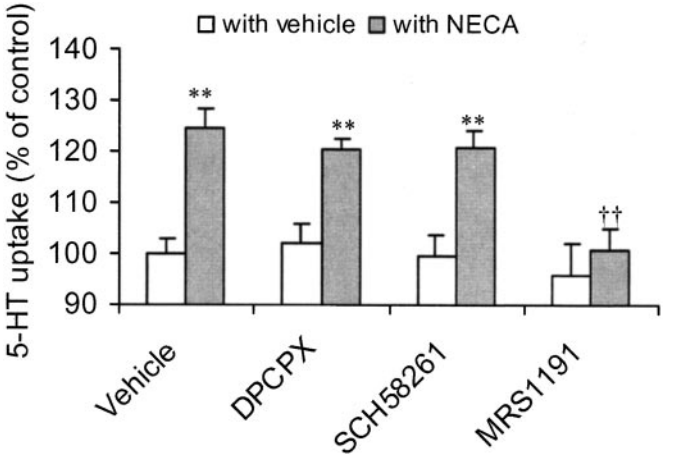

Fig. 2. Effect of AR antagonists on NECA-induced 5-HT uptake. RBL-2H3 cells were treated for 20 min with the antagonist or vehicle (□) or preincubated with antagonists (▨) for 10 min before adding NECA (1 μM) for 10 min before transport assay. Antagonists for the A1 (DPCPX, 1.0 μM) or A_{2A} (SCH58261, 10 nM) receptor did not affect NECA stimulation of 5-HT uptake. A3 (MRS1191, 1 μM) antagonist completely blocked NECA-induced 5-HT uptake. Values are expressed as mean values (n = 4) ± S.E.M. **, $p < 0.01$ versus vehicle; †, $p < 0.05$ and ††, $p < 0.01$ versus NECA.

Ca^{2+} requirement for NECA stimulation of SERT is needed only upstream of NOS activation.

Active Guanyl Cyclase and PKG Are Required for NECA Stimulation of SERT. To further validate this model, we next tested two soluble guanyl cyclase inhibitors, ODQ and LY83583 (Fig. 4A). ODQ, a NO-sensitive soluble guanyl cyclase inhibitor, blocked NECA's effect on 5-HT transport without affecting basal uptake. Consistent with prior findings (Miller and Hoffman, 1994), LY83583 also blocked the effect of NECA, although it also inhibited basal uptake of 5-HT, suggestive of possible direct effects on SERT or its regulation independent of guanyl cyclase. Regardless, these findings lend further support to the idea that 5-HT transport is increased by NECA through the enhancement of cytosolic guanyl cyclase activity in keeping with findings of NECA-stimulated cGMP production by RBL-2H3 cells (Miller and Hoffman, 1994).

The effects of cGMP are mediated through a number of downstream effectors, including PKG, cGMP-activated cGMP phosphodiesterase, and cyclic nucleotide-gated ion channels. We found that the increase in 5-HT uptake induced by NECA was fully blocked by pretreatment of cells with H8, a selective PKG inhibitor (Fig. 4B). Furthermore, with H8, we achieved complete inhibition of SERT stimulation triggered by hydroxylamine, 8-bromo-cGMP, sildenafil, and NECA plus sildenafil. These findings further link NECA actions to the consequences of NOS stimulation and cGMP production and support the contention that catalytically active PKG is essential for the SERT stimulatory pathway under study.

Involvement of p38 MAPK and PP2A in AR-Stimulated 5-HT Uptake. In addition to PLC activation, multiple MAP kinases are activated after AR stimulation (Feoktistov et al., 1999). We found that NECA stimulation of 5-HT up-

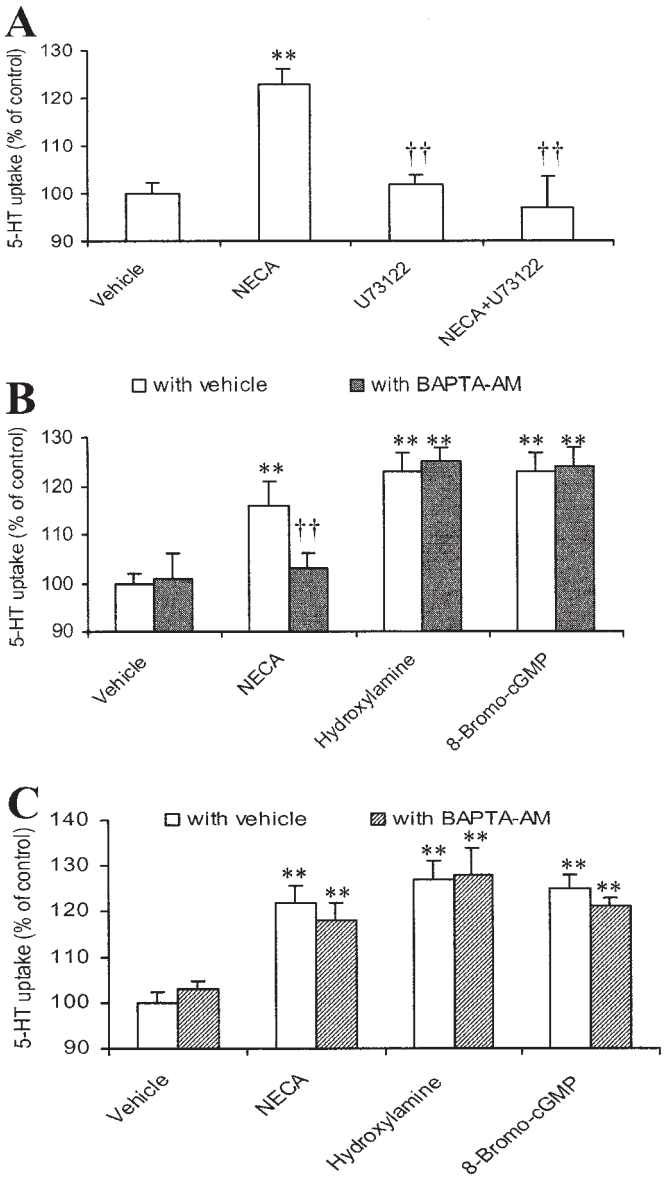

Fig. 3. Effect of PLC inhibition and Ca^{2+} chelation on NECA-induced 5-HT uptake. A, RBL-2H3 cells were pretreated with vehicle or the PLC inhibitor U73122 (10 μM) 10 min before adding NECA (1 μM) for a total of 20 min followed by 5-HT transport assay. U73122 on its own did not impact 5-HT uptake but fully inhibited NECA-induced 5-HT transport stimulation. B, Cells were preincubated with BAPTA-AM (10 μM) in the absence of extracellular Ca^{2+} for 10 min before adding NECA (1 μM), hydroxylamine (100 μM), or 8-bromo-cGMP (10 μM) for 10 min followed by transport assay. NECA stimulation of 5-HT uptake was blocked by BAPTA-AM, whereas stimulation induced by hydroxylamine and 8-bromo-cGMP was unaffected. C, external Ca^{2+} can override the effect of intracellular Ca^{2+} chelation. Cells were incubated with BAPTA-AM (10 μM) in the presence of extracellular Ca^{2+} 10 min before adding NECA (1 μM). Under these conditions, BAPTA-AM does not affect the stimulation of 5-HT uptake by NECA nor the modulation of hydroxylamine and 8-bromo-cGMP effects. Values are expressed as mean values (n = 5) ± S.E.M. **, $p < 0.01$ versus vehicle; ††, $p < 0.01$ versus NECA.

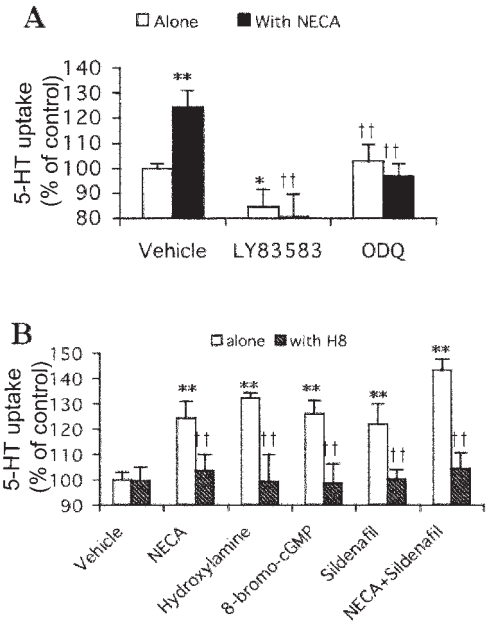

Fig. 4. Guanyl cyclase and PKG activities are required for NECA stimulation of SERT. A, effect of guanyl cyclase inhibitors on NECA stimulation of 5-HT transport. RBL-2H3 cells were incubated with vehicle or guanyl cyclase inhibitors (LY83583, 10 μM; or ODQ, 10 μM) alone for 20 min or for 10 min followed by application of NECA (1 μM) for a total of 20 min. Both LY83583 and ODQ blocked NECA-induced 5-HT uptake. In contrast to LY83583, ODQ did not affect basal uptake. Activities are expressed as a percentage of vehicle controls (n = 4) ± S.E.M. *, $p < 0.05$ and **, $p < 0.01$ versus vehicle; ††, $p < 0.01$ versus NECA. B, PKG-dependence of hydroxylamine, 8-bromo-cGMP, NECA, and sildenafil stimulation of 5-HT transport. Cells were pretreated with vehicle or the PKG inhibitor H8 (0.1 μM) 10 min before adding each of the modulators (▨) for an additional 10 min. Modulator concentrations used were 1 μM NECA, 100 μM hydroxylamine, 10 μM 8-bromo-cGMP, and 4 μM sildenafil. Values are expressed as mean values (n = 5) ± S.E.M. **, $p < 0.01$ versus vehicle; ††, $p < 0.01$ versus corresponding modulators alone.

take in RBL-2H3 was insensitive to the mitogen-activated protein kinase kinase inhibitor PD98059 (data not shown). Preincubation of RBL-2H3 cells with SB203580 (10 μM), a p38 MAPK inhibitor (Cuenda et al., 1995), markedly blocked SERT stimulation by NECA, hydroxylamine, or 8-bromo-cGMP (Fig. 5A). Similar inhibition was found with SB202190 (10 μM), a distinct p38 MAPK inhibitor (data not shown). Next, we established whether activation of p38 MAPK is triggered by NECA in RBL-2H3 cells, under our assay conditions, monitoring the extent of p38 MAPK phosphorylation using total and phosphospecific antibodies. As shown in Fig. 5B, blots of RBL-2H3 extracts revealed basal levels of p38 MAPK phosphorylation that could be enhanced by the p38 MAPK activator anisomycin (1 μM). Treatment of cells with NECA also significantly increased p38 MAPK phosphorylation with no change in total kinase levels (Fig. 5B). As with NECA-induced 5-HT uptake stimulation, incubation of cells with the p38 MAPK inhibitor SB203580 before NECA treatments abolished NECA-induced p38 MAPK phosphorylation.

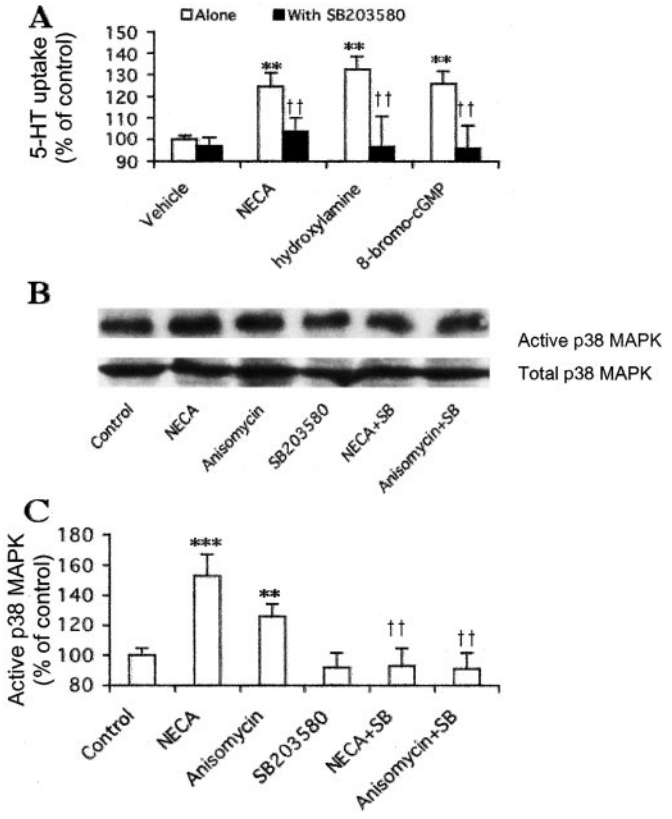

Fig. 5. Evidence for a role of p38 MAPK in SERT stimulation by NECA. A, the p38 MAPK inhibitor SB203580 blocks NECA, hydroxylamine, and 8-bromo-cGMP stimulation of 5-HT uptake. Cells were preincubated with vehicle or SB203580 (10 μM) 10 min before adding modulators (1 μM NECA, 10 μM hydroxylamine, or 10 μM 8-bromo-cGMP; ■), or treated with the vehicle/modulators alone (□). SB203580 blocked 5-HT uptake stimulation without a significant effect on basal 5-HT uptake. B, NECA treatment of RBL-2H3 cells triggers activation of p38 MAPK (top) without affecting total kinase (bottom) levels. Cells were treated as in A except that they were subsequently processed for activated p38 MAPK immunoblots as described under *Materials and Methods*. NECA stimulated p38 MAPK as did anisomycin (as positive control). SB203580 blocked both NECA or anisomycin-stimulated increase of activated p38 MAPK. C, average values of activated p38 MAPK immunoblots from densitometry of experiments as described in B (n = 3). Values are expressed as the mean ± S.E.M. **, $p < 0.01$ versus vehicle; ††, $p < 0.01$ versus modulator in the absence of SB203580.

ARs have recently been found to trigger the translocation and activation of PP2A via a p38 MAPK-sensitive pathway (Liu and Hofmann, 2003). The ability of PP1/2A inhibitors okadaic acid and calyculin A to trigger SERT phosphorylation and down-regulation (Ramamoorthy et al., 1998) and the findings that SERT physically associates with PP2A catalytic subunit (PP2Ac) (Bauman et al., 2000) encouraged us to examine the sensitivity of NECA effects to PP2A inhibition. When we preincubated cells in calyculin A under conditions that fail to impact basal 5-HT transport (Fig. 6), we nonetheless achieved a complete blockade of NECA, hydroxylamine, and 8-bromo-cGMP stimulation of SERT activity.

AR Stimulation of RBL-2H3 Cells Triggers an Increase in Surface SERT Binding Sites. AR stimulation of 5-HT uptake could involve alterations in transporter surface trafficking, an enhancement of catalytic function, or both. Levels of SERT protein in RBL-2H3 cells are not high enough to use conventional antibody paradigms to detect SERT redistribution in response to receptor stimulation. To establish whether NECA-induced 5-HT uptake is paralleled by surface changes in SERT, we implemented a whole-cell, radioligand binding paradigm using the cocaine analog [^{125}I]RTI-55. RTI-55 is membrane-permeant and should label both surface and intracellular sites. We used either paroxetine (10 μM) as displacer to monitor total (surface plus intracellular) specific binding or the hydrophilic ligand 5-HT (100 μM) as displacer to define surface binding. Our assay temperature (4°C) was chosen to limit 5-HT uptake inside the cell as well as transporter exocytosis/endocytosis. We have successfully applied these same conditions in assessing surface density of NET proteins in SK-N-SH cells, in this case monitoring norepinephrine-sensitive [^{3}H]nisoxetine binding (Apparsundaram et al., 2001). Initial analysis of paroxetine-displaceable label-

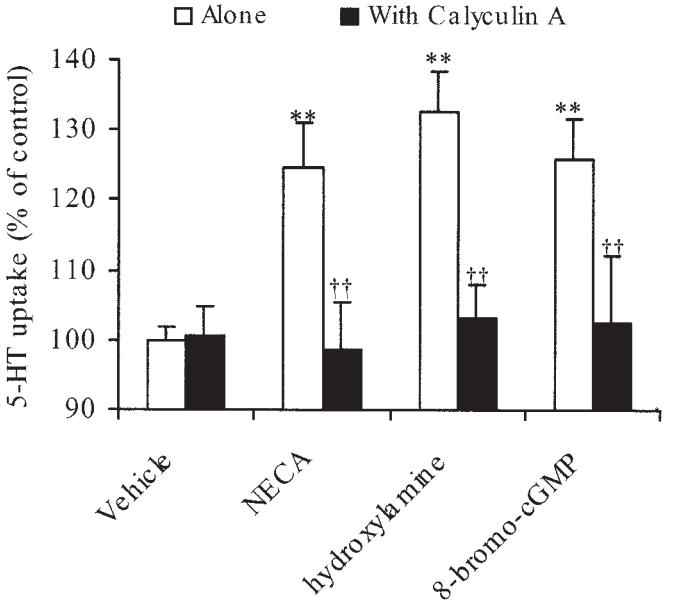

Fig. 6. The PP1/2A inhibitor calyculin A blocks stimulation of 5-HT uptake. RBL-2H3 cells were preincubated with vehicle or calyculin A (0.1 μM) 10 min before the addition of modulators (■) for a subsequent 10 min before transport assay. Modulator concentrations used were 1 μM NECA, 10 μM hydroxylamine, and 10 μM 8-bromo-cGMP. Calyculin A blocked the 5-HT uptake induced by NECA, hydroxylamine, or 8-bromo-cGMP without affecting the basal uptake. Values are expressed as mean values (n = 5) ± S.E.M. **, $p < 0.01$ versus vehicle; ††, $p < 0.01$ versus modulator in the absence of calyculin A.

ing (Fig. 7, A-C) revealed that [^{125}I]RTI-55 bound to cells in a staurable manner, well fit to a single-site model (K_d = 0.44 ± 0.10 nM). Approximately 30% of this binding was displaceable by incubation with 5-HT. The 5-HT–defined [^{125}I]RTI-55 binding has the same K_d (0.43 ± 0.17 nM) as total [^{125}I]RTI-55 binding. Using the 5-HT–defined B_{max} as a quantitative estimate for surface density, we calculate a maximal turnover rate (V_{max}/B_{max}) at 37°C for SERT to be 0.83 molecules of 5-HT transported per transporter per second.

To ascertain whether the whole-cell, 5-HT–displaceable

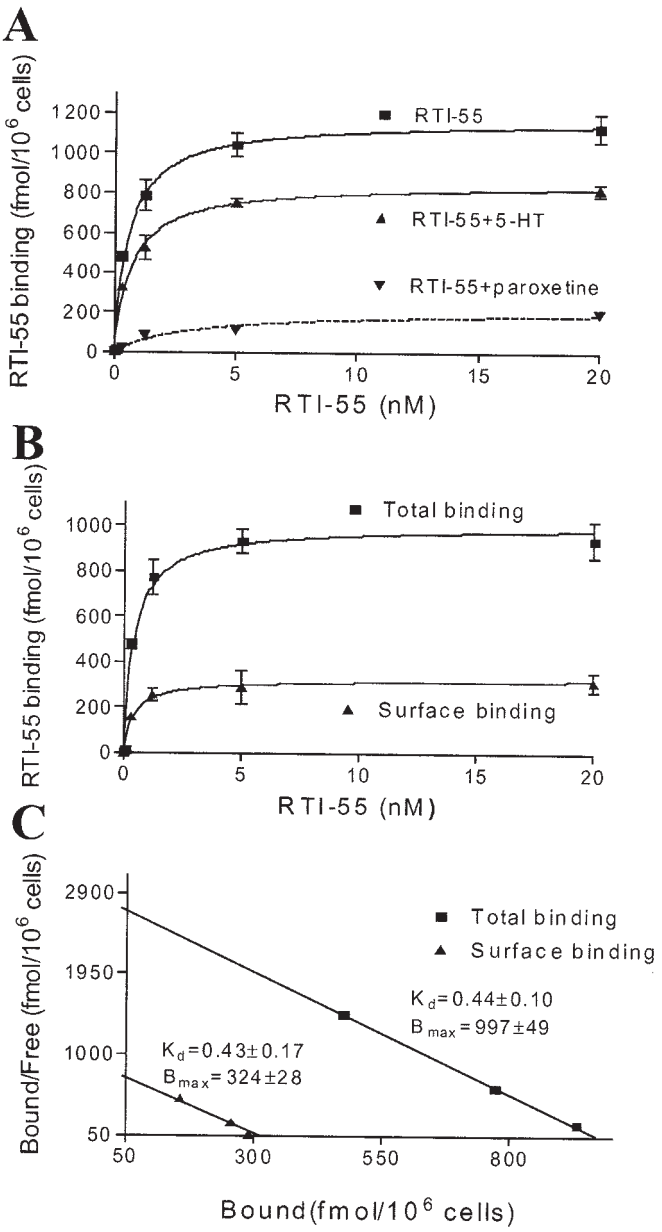

Fig. 7. Saturation analysis of [^{125}I]RTI-55 binding in RBL-2H3 cells. Cells were preincubated with 100 μM 5-HT or 10 μM paroxetine for 5 min on ice before adding [^{125}I]RTI-55. A, 5-HT displaces ~30% of [^{125}I]RTI-55 binding, whereas ~90% of total binding is displaced by paroxetine. B, total specific binding as defined by subtracting the binding of [^{125}I]RTI-55 achieved in the presence of paroxetine alone from total binding, and surface-specific binding as calculated by subtracting [^{125}I]RTI-55 binding in the presence of 5-HT from that of [^{125}I]RTI-55 alone. C, Scatchard transformation of the total and surface [^{125}I]RTI-55 binding data from A and B above. Surface density (B_{max}) amounts to ~30% of total binding, whereas both surface and total binding exhibit an equivalent K_d.

[^{125}I]RTI-55 binding behaves as expected if indeed it represents the density of surface SERT protein, we examined the effects of phorbol ester β-PMA on binding activity (Fig. 8A). β-PMA is well known to trigger SERT internalization in various models and reduces SERT V_{max} but not total [^{3}H]paroxetine binding in RBL-2H3 cells (Miller and Hoffman, 1994). Indeed, we measured a significant (47%) reduction in surface labeling after short-term (30 min) treatment of cells with 1.0 μM β-PMA (Fig. 8A). In contrast, when cells were treated with NECA, the density of [^{125}I]RTI-55 surface binding increased (Fig. 8A). Saturation analysis of the NECA effects on 5-HT–sensitive [^{125}I]RTI-55 binding revealed a significant increase in B_{max}, with no increase in [^{125}I]RTI-55 K_d value (Fig. 8B). In contrast, no change in paroxetine-defined [^{125}I]RTI-55 binding was observed after NECA treatment. We observed similar increases in surface [^{125}I]RTI-55 binding with the A_3 AR agonist IB-MECA ± sildenafil (Fig. 8C). As with transport assays, both IB-MECA and sildenafil enhanced surface binding activity, with a greater increase observed with their coapplication. MRS1191 fully blocked the stimulatory effect on binding of IB-MECA alone, whereas it partially attenuated the binding increase of IB-MECA plus sildenafil, in keeping with the fact that sildenafil can augment basal cGMP levels downstream of AR activation. Consistent with this idea, the PKG inhibitor H8 completely blocked the elevation in 5-HT–sensitive [^{125}I]RTI-55 triggered by IB-MECA/sildenafil (Fig. 8C). In agreement with findings by Miller and Hoffman (1994), total levels of SERT, as defined by paroxetine displacement of [^{125}I]RTI-55 binding, do not change in response to these agents, including NECA (Fig. 8D).

Increases in SERT surface density in RBL-2H3 cells could arise from increased fusion rates of intracellular SERT vesicles or decreases in endocytosis of rapidly recycling surface carriers. To investigate these possibilities in RBL-2H3 cells, we inactivated surface SERTs with the membrane-impermeant, cysteine-selective modifying reagent MTSET (Chen et al., 1997). Under our conditions (10 mM MTSET, 10-min treatment at room temperature), we achieved 78 ± 4.5% inactivation of 5-HT transport. We then re-examined NECA modulation, comparing MTSET with vehicle-pretreated cultures. If NECA effects arise from a slowing of SERT endocytosis, removing a majority of carriers should blunt the NECA effect. Instead, we found that NECA was still able to stimulate SERT activity in MTSET-treated cultures. Indeed, measured as a percentage of the basal level of activity, the extent of NECA stimulation actually doubled. If the ARs are protected with NECA before the MTSET treatment, the NECA-induced stimulation is even greater (Fig. 9). These findings suggest that NECA-triggered increases in SERT surface density most probably arise from the insertion into the plasmalemma of intracellular SERTs.

AR Stimulation of SERT Also Involves a p38 MAPK-Dependent Catalytic Activation. As noted above, NECA stimulation of RBL-2H3 cells leads to enhanced p38 MAPK phosphorylation, and SERT stimulation can be blocked by specific p38 MAPK inhibitors. It is possible that p38 MAPK acts to facilitate SERT translocation to the plasma membrane. On the other hand, as suggested from studies of insulin-responsive GLUT4 glucose transporters (Sweeney et al., 1999) and NET (Apparsundaram et al., 2001) proteins, p38 MAPK may play a more critical role in catalytic activation.

To assess these possibilities, we repeated our binding studies after NECA treatment in the presence or absence of SB203580 at a concentration (10 μM) that fully blocks NECA stimulation of 5-HT transport (Fig. 10). We found that unlike the sensitivity of 5-HT uptake stimulation to p38 MAPK inhibition, surface SERT density elevations were unaffected by SB203580. These findings indicate that, in RBL-2H3 cells, activated p38 MAPK plays a role in catalytic activation of SERT rather than supporting the transporter's surface trafficking but that these two processes are triggered by a PKG-dependent mechanism to set levels of SERT as monitored in 5-HT transport assays.

ARs Enhance SERT Activity in Receptor/Transporter Cotransfected CHO Cells via Elevations of Surface SERT Protein. As noted above, SERT protein levels in RBL-2H3 are too low for biochemical assessments of SERT regulation. To develop a system that would permit greater flexibility in molecular manipulations of the AR/SERT signaling pathway and to validate surface density changes as a critical step in SERT stimulation, we sought to reconstitute the interaction using cotransfected CHO cells. It is noteworthy that CHO cells are known to support both cGMP and PKG-mediated signaling pathways (Pfeifer et al., 1995) and have been used previously for studies of heterologously expressed ARs (Schulte and Fredholm, 2000). CHO cells without transfected SERT do not show evidence of antidepressant-sensitive 5-HT transport (data not shown). For cotransfection studies, we largely focused on A_3 cDNA because the antagonist studies described above pointed to a more dominant role for this receptor in SERT stimulation in RBL-2H3 cells. As predicted by RBL-2H3 studies, A_3 receptors cotransfected with SERT support a significant increase in 5-HT uptake triggered either by IB-MECA (Fig. 11A) or NECA (Fig. 12A) and which can be potentiated by sildenafil. Cells expressing SERT but lacking A_3 receptor were insensitive to agonist treatments (data not shown). It is important to note that the A_3 antagonist MRS1191 attenuated the A_3 stimulation of SERT, whereas the effect of sildenafil was AR antagonist-insensitive (Fig. 11A). Moreover, the entire effect of IB-MECA/sildenafil was blocked by the PKG inhibitor H8. In conclusion, as seen with RBL-2H3 cells, SERT stimulation achieved in A_3 AR/SERT-cotransfected CHO cells was blocked by the p38 MAPK inhibitor SB203580. These findings suggest that a process similar to that at work in RBL-2H3 cells can produce AR-stimulated SERT activity in a heterologous model system.

A_{2B} receptors are also present in RBL-2H3 cells and could cosignal with A_3 AR to modulate SERT activity. When SERT was cotransfected with A_{2B} cDNA, we also found that NECA stimulated 5-HT transport (control, 100 ± 5%; A_{2B}, 114 ±

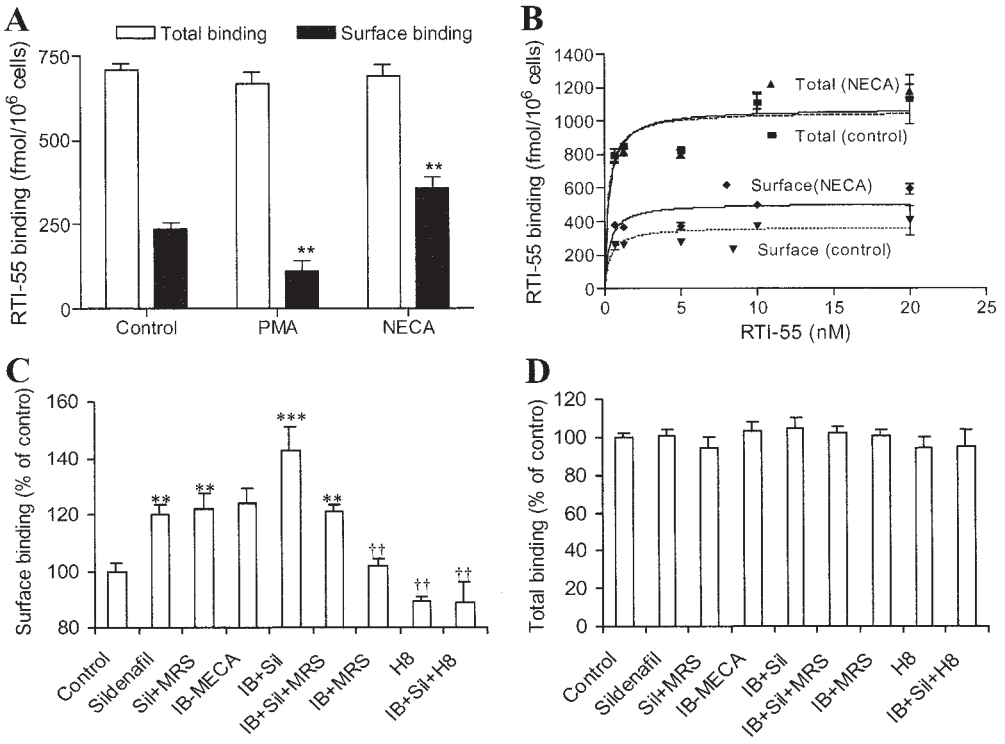

Fig. 8. Stimulation by NECA/sildenafil induces an increase in SERT surface density. A, RBL-2H3 cells were preincubated with vehicle or β-PMA (1 μM) for 30 min or NECA (1 μM) for 10 min before measurement of 5-HT–sensitive [^{125}I]RTI-55 binding. β-PMA triggered a significant reduction in binding, whereas NECA induced a significant increase in binding. Neither pretreatment influenced total binding. B, saturation analysis of NECA effects on [^{125}I]RTI-55 binding in RBL-2H3 cells. Cells were treated with NECA or vehicle for 10 min before the binding assay. In vehicle-treated cells, the Bmax (fmol/10^6 cells):Kd (nM) for the total binding is 1055 ± 61:0.26 ± 0.12; for the surface binding, 367 ± 32:0.37 ± 0.20. In NECA treated cells, the B_{max}:K_d for the total binding is 1072 ± 55:0.29 ± 0.10 and for the surface binding is 508 ± 31**:0.32 ± 0.13. C and D, stimulators of 5-HT uptake increase 5-HT–displaceable [^{125}I]RTI-55 surface binding with no change in total SERT density. Cells were treated with the indicated modulators followed by whole-cell [^{125}I]RTI-55 binding assays conducted in the presence or absence of 5-HT (C) or paroxetine (D). No change was observed in paroxetine-displaceable binding, whereas significant alterations were seen in 5-HT–displaceable binding. IB-MECA (1 μM) and sildenafil as well as the combination induced an increase in 5-HT–displaceable [^{125}I]RTI-55 whole-cell binding. H8, a PKG inhibitor, blocked this increase. MRS1191 (1 μM) blocked the effect of IB-MECA without affecting that of sildenafil. Values are expressed as mean values (n = 3) ± S.E.M. **, $p < 0.01$; $p < 0.001$ versus vehicle; ††, $p < 0.01$ versus modulator.

6%). In contrast, no stimulation was observed with A_{2A} receptor cotransfection (103 ± 5% of control). For completeness, transfection of A_1 receptor cDNA was examined. A_1 receptors are not native to RBL-2H3 cells but couple through pathways similar to A_{2B} and A_3 (Feoktistov and Biaggioni, 1997; Fredholm et al., 2000). In A_1AR/SERT cotransfected CHO cells, we found that *R*-PIA (100 nM), an A_1 receptor agonist, as well as NECA (1 μM), stimulated an increase in 5-HT uptake (122 ± 3% and 118 ± 2% relative to control conditions, respectively). Both effects were completely blocked by the specific A1 antagonist DPCPX (1 μM) (data not shown). These findings indicate that CHO cells provide a hospitable environment for reconstitution of AR-mediated increases in SERT activity and that signaling pathways supporting SERT regulation are probably not unique to the RBL-2H3 environment.

We next sought to determine whether AR stimulation of SERT activity in CHO cells was accompanied by changes in cell-surface density of SERT protein. First, we implemented the surface-labeling paradigm previously applied using [^{125}I]RTI-55 in RBL-2H3 cells. As in RBL-2H3 cells, stimuli that trigger an increase in 5-HT uptake, including IB-MECA, sildenafil, and their combination, increased proportionately 5-HT–displaceable [^{125}I]RTI-55 binding (Fig. 11B). Moreover, these changes in surface SERT labeling were sensitive to A_3 AR and PKG antagonists, just as in RBL-2H3 cells. Next, we implemented a membrane-impermeant biotinylating reagent to explore changes in surface SERT density (Qian et al., 1997; Ramamoorthy and Blakely, 1999). Using the biotinylation paradigm (Fig. 11, C and D), we found that IB-MECA and/or sildenafil triggers an increase in surface SERT protein commensurate with 5-HT transport and [^{125}I]RTI-55–labeling studies. As noted for uptake stimulation, the A3 antagonist MRS1191 blocked the increases in SERT density, whereas sildenafil, acting downstream of AR stimulation, augmented SERT density in an MRS1191-insensitive manner. Finally, the PKG inhibitor H8 blocked the effect of both the A_3 agonist and sildenafil on SERT surface expression.

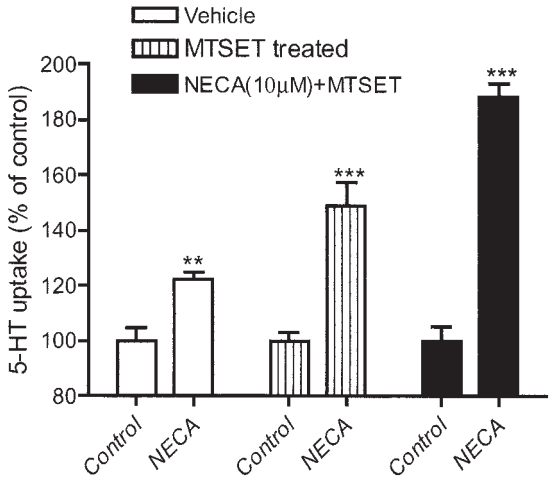

Fig. 9. MTSET inactivation of surface SERTs fails to block NECA stimulation of SERT. RBL-2H3 cells were preincubated with vehicle (□ and ▥) or NECA (■) on ice for 10 min before washing followed by incubation with MTSET (10 mM, ▥ and ■) for 10 min before NECA treatments. Values are expressed as mean values (n = 3) ± S.E.M. ⋆⋆, $p < 0.01$; ⋆⋆⋆, $p < 0.001$ versus respective control. Absolute values for three controls (□, ▥, and ■) are 112.56 ± 7.53, 25.40 ± 3.56, and 24.48 ± 3.82 fmol/well/min, respectively.

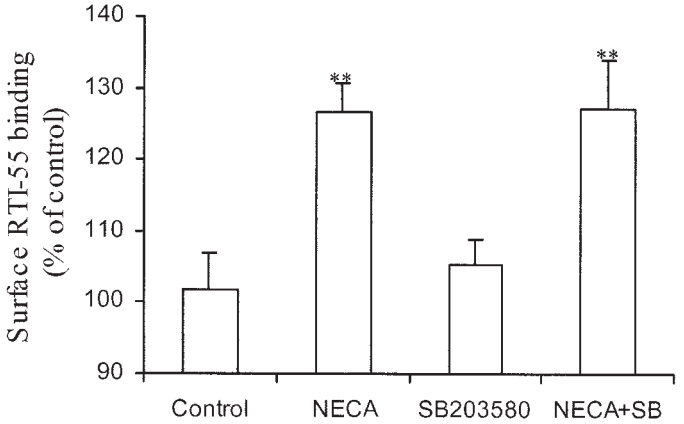

Fig. 10. p38 MAP kinase inhibition fails to block NECA stimulation of SERT surface binding. RBL-2H3 cells were incubated for 10 min with SB203580 before NECA treatments. Values are expressed as mean values (n = 3) ± S.E.M. ⋆⋆, $p < 0.01$ versus control.

Next we asked whether p38 MAPK inhibition, which blocks AR-induced up-regulation of transport activity, would also block the increase in surface SERT protein or whether, like RBL-2H3 cells, these two events can be dissociated. When AR stimulation was repeated in the presence of SB203580, we again obtained full blockade of uptake stimulation (Fig. 12A). However, biotinylation studies revealed no antagonism of A_3 AR-induced increases in SERT surface protein (Fig. 12, B and C). As in RBL-2H3 cells, SB203580 under these conditions does not impact basal SERT surface expression levels, suggesting that carriers already targeted to the plasma membrane before AR stimulation do not require sustained p38 MAPK activation, at least over the short time period of our incubations. These results provide strong support for the contention, first developed in our RBL-2H3 cell studies, that AR-mediated enhancement of 5-HT uptake occurs as a consequence of a two-step process involving both an increase in SERT surface density and a separate process of catalytic activation, achieved through the activation of a p38 MAPK-dependent pathway.

Discussion

A desire to investigate SERT regulation in native systems led us to explore in greater detail the ability of AR-linked stimuli to impact 5-HT uptake in RBL-2H3 cells, as first noted by Miller and Hoffman (1994). Elucidation of pathways supporting receptor-stimulated SERT up-regulation could be of great benefit in the pharmacological modulation of psychiatric and autonomic disorders and help to identify candidate pathways supporting disease risk/progression. Our findings regarding AR stimulation of SERT integrating previous findings are provided in schematic form in Fig. 13. ARs are members of the G-protein coupled receptor superfamily. In vivo, there is evidence that both release and reuptake of 5-HT can be regulated by ARs (Miller and Hoffman, 1994; Okada et al., 1999). In the case of 5-HT transport, Miller and Hoffman (1994) suggested that activation of A_3 receptors by NECA in RBL-2H3 cells leads to increased SERT function. In addition to A_3 receptors, RBL-2H3 cells express A_{2A} and A_{2B} to a lesser extent, but seem to lack A_1 receptors (Feoktistov and Biaggioni, 1997). We found that NECA-stimulated 5-HT up-

take in RBL-2H3 cells was blocked by A_3 receptor antagonists (MRS1191) but not by A_{2A} antagonist SCH58261. However, in CHO cells cotransfected with A_1 AR and SERT cDNAs, NECA and *R*-PIA, a specific A_1 agonist, induce an increase in 5-HT transport that can be blocked by the A_1 antagonist DPCPX. NECA also stimulated 5-HT uptake in CHO cells cotransfected with A_{2B} AR and SERT cDNAs, although to a lesser extent than cotransfected A_3 ARs. These results indicate that signaling pathways available to A_1, A_{2B}, and A_3 receptors can trigger an up-regulation of SERT activity, although A_3 is the most likely physiologically relevant target in RBL-2H3 cells.

ARs are known to signal to downstream effectors through inhibitory Gi/o (A_1 and A_3), stimulatory Gs (A_{2A} and A_{2B}), and Gq proteins, with the latter pathway coupled to PLC activation (A_1, A_{2B}, and A_3 receptors) (Feoktistov and Biaggioni, 1997). Miller and Hoffman (1994) proposed that NECA stimulated an elevation of intracellular Ca^{2+}, leading to activation of NOS and subsequent cGMP production and PKG activation. SERT activity and regulation in native preparations, including platelets (Turetta et al., 2002) and synaptosomes (Nishio et al., 1995; Ansah et al., 2003), has been reported to be sensitive to manipulation of intracellular Ca^{2+}, although precise mechanisms have yet to be defined. We found that chelation of Ca^{2+} with BAPTA-AM blocked the ability of NECA to stimulate SERT activity. Although NECA's effect was blocked, chelation of intracellular Ca^{2+} did not affect 5-HT uptake induced by hydroxylamine, an NO donor, or by sildenafil, a PDE5 inhibitor. These data led us to propose that the effect of Ca^{2+} occurs upstream of the production of NO and cGMP, NOS is a Ca^{2+}/calmodulin-dependent enzyme, and Ca^{2+} could be required to activate NOS because calmodulin antagonists negatively impact SERT activity in placental cells (Jayanthi et al., 1994), although at present, there is no indication that the latter effect derives from a block of NOS activation. Regardless, elevation of intracellular Ca^{2+} is well known to be a stimulus to activate NOS, and NO produced from NOS activation stimulates soluble guanyl cyclase. Of particular note, SERT has recently been found to colocalize with NOS in raphe neurons and axonal projections. (Simpson et al., 2003). Indeed, Miller and Hoffman (1994) and our group (C.-B. Zhu, W. A. Hewlett, and R. D. Blakely, unpublished observations) have found that N^{ω}-nitro-L-arginine methyl ester, a NOS inhibitor, effectively abolishes NECA-induced stimulation of SERT.

The cGMP subsequently produced by NO-stimulated guanyl cyclase can activate PKG and lead to phosphorylation of PKG substrates. In this regard, SERT itself is known to be

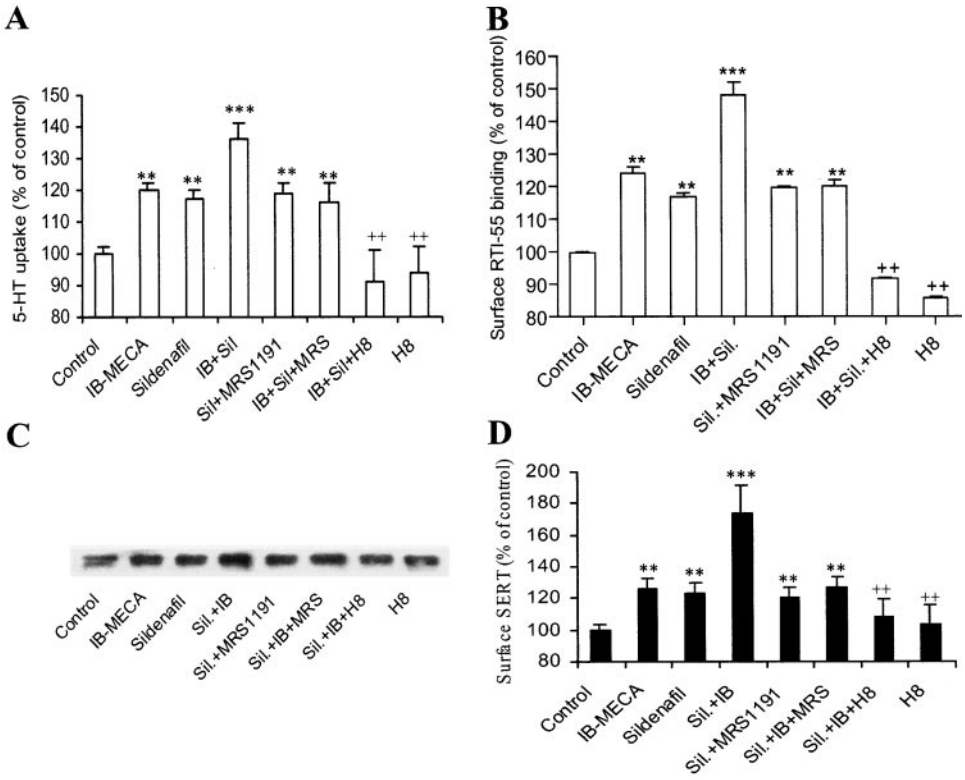

Fig. 11. Elevation of 5-HT transport and SERT surface expression by the A_3 agonist IB-MECA in SERT/A_3 AR-cotransfected CHO cells. Cells were cultured and transfected with transporter/receptor cDNAs as described under *Materials and Methods*. Cells were cultured overnight after the transfection before either 5-HT uptake assays (A), [^{125}I]RTI-55 binding assay (B), or cell surface biotinylation assays (C and D). A, cells were treated individually or jointly with IB-MECA (1 μM) or sildenafil (4 μM) or with vehicle for 10 min before transport assays. The stimulatory effect of IB-MECA was further enhanced by sildenafil. H8 (1.0 μM) blocked the 5-HT uptake induced by either IB-MECA or sildenafil or their combination. The A_3 antagonist MRS1191 (1.0 μM) blocked the effect of IB-MECA but not that of sildenafil. B, cells were pretreated with modulators as in A followed by the determination of 5-HT–sensitive [^{125}I]RTI-55 binding assays on ice. C, representative Western blot of biotinylated surface fractions of SERT/receptor cotransfected cells. Cells were pretreated with modulators as in A followed by biotinylation and surface fraction isolation/immunoblotting as described under *Materials and Methods*. D, average quantification of signals ($n = 3$) from biotinylation experiments are presented and are expressed relative to levels detected in untreated samples. Values are expressed as mean values ± S.E.M. **, $p < 0.01$ and ***, $p < 0.001$ versus control; ††, $p < 0.01$ versus modulator in the absence of H8. Both IB-MECA and sildenafil treatments alone induced a significant increase in surface-expressed SERT protein/[^{125}I]RTI-55 binding. When IB-MECA and sildenafil were applied simultaneously, an enhanced increase was noted. All surface elevations in SERT protein/[^{125}I]RTI-55 binding were blocked by pretreatment with the PKG inhibitor H8.

phosphorylated after treatment of cells with 8-bromo-cGMP (Ramamoorthy et al., 1998). This second-messenger analog triggers in RBL-2H3 cells a comparable stimulation of 5-HT uptake as observed with NECA. Consistent with a primary importance of a PKG pathway in AR-mediated activation of SERT, ODQ, a soluble guanyl cyclase inhibitor, blocked NECA-induced 5-HT uptake. Moreover, the AR-mediated increase in 5-HT uptake was fully sensitive to the PKG inhibitor H8 and could be potentiated by the selective inhibitor of PDE5, sildenafil. We also observed stimulation by sildenafil alone, indicating basal cGMP production in our system. Indeed, H8 blocked the effect of both NECA and sildenafil as well when the two agents were applied together. Our studies thus identify a novel PKG-dependent consequence of sildenafil blockade of PDEs in promoting enhanced 5-HT transport. Sildenafil has recently been shown to exert central effects (Milman and Arnold, 2002), and the cGMP-specific PDE targeted by sildenafil, PDE5, is expressed in the brain, including the cerebral cortex, hippocampus, and basal ganglia (Garthwaite and Boulton, 1995). Whether the activities we have described in vitro influence SERT and central serotonergic transmission will be the target of future studies.

The sensitivity of SERT to PKG-mediated regulation noted here and in our previous phosphorylation studies (Ramamoorthy et al., 1998) warrants further consideration of the direct regulatory potential of this pathway in modulating SERT trafficking and activity. In this regard, we note with interest the recent report from Rudnick's laboratory (Kilic et al., 2003), revealing that a naturally occurring SERT coding polymorphism (Glatt et al., 2001) seems to be refractory to PKG-linked SERT stimulation. The polymorphism, which results in a conservative substitution at an intramembrane hydrophobic residue (I425V), may alter SERT conformation to mimic the state achieved after PKG-dependent SERT phosphorylation. That this mutation is functionally relevant is suggested by a recent report describing two pedigrees in which a high percentage of carriers of this variant suffer from obsessive-compulsive disorder (Ozaki et al., 2003). Studies examining the requirement of SERT PKG phosphorylation sites for NECA stimulation are underway.

Using cell-surface radioligand binding and membrane-impermeant biotinylating paradigms in RBL-2H3 cells and transfected CHO cells, respectively, we gathered evidence that PKG-dependent stimulation of SERT downstream of AR activation arises from an increase in SERT surface density. Miller and Hoffman (1994) also used whole-cell binding to

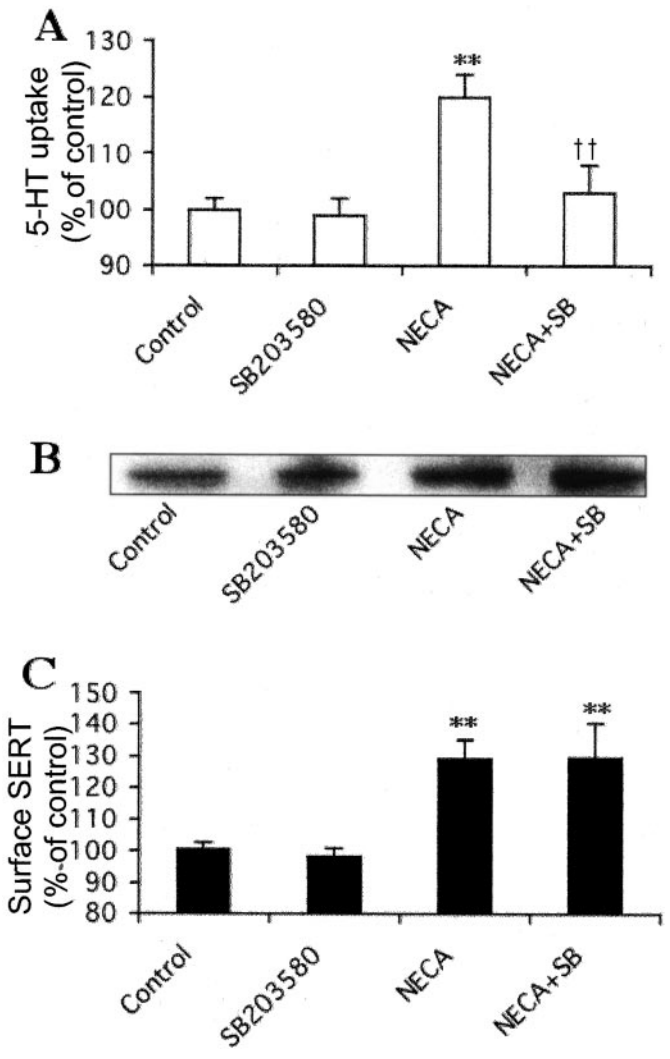

Fig. 12. p38 MAPK inhibition does not attenuate NECA-stimulated SERT surface expression in SERT/AR-cotransfected CHO cells. Cells were transfected with A_3 receptor and human SERT cDNA as described under *Materials and Methods*. Experimental conditions are the same as those described under Fig. 11. A, 5-HT transport assays. NECA (1 μM) induced an increase of 5-HT transport; SB203580 (10 μM) blocked NECA's effect without affecting basal 5-HT transport activity. B and C, impact of SB203580 on changes in SERT surface protein as detected by biotinylation and immunoblot. Application of NECA (1 μM) led to an elevation of surface SERT expression. SB203580 (10 μM) did not affect either NECA-stimulated or basal surface expression of SERT. B, representative SERT immunoblot of biotinylated fractions. C, average quantification of signals from four biotinylation experiments. Values are expressed as mean values ($n = 4$) ± S.E.M. **, $p < 0.01$ versus control; ††, $p < 0.01$ versus NECA alone.

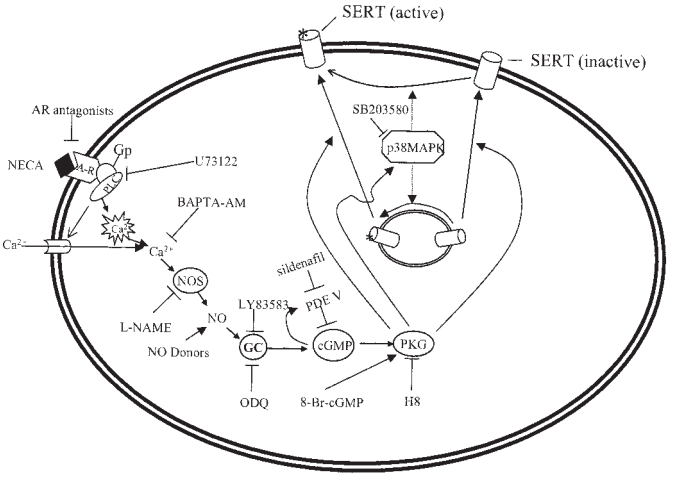

Fig. 13. Schematic illustration of signaling pathways implicated in AR-triggered up-regulation of SERT in RBL-2H3 cells and receptor/transporter-cotransfected CHO cells. Activation of ARs (such as with NECA) stimulates Gq-linked PLC, which triggers an increase of intracellular Ca^{2+} both by the release of internal Ca^{2+} stores and by Ca^{2+} influx. The increased Ca^{2+} stimulates NOS to produce NO that subsequently activates the soluble guanyl cyclase, producing elevated levels of cGMP. cGMP activates PKG but is negatively regulated by sildenafil-sensitive PDE 5. NECA treatments also lead to activation of p38 MAPK, and p38 MAPK inhibitors block NECA stimulation of 5-HT uptake. We hypothesize that p38 MAPK activation may occur via PKG stimulation. Stimulation of 5-HT uptake involves enhanced surface density of SERT but also requires catalytic activation via a p38 MAPK-linked pathway. Activation may target transporters before or after insertion. *, activated SERT.

evaluate changes in SERT protein levels. These authors found no change in specific SERT radioligand binding after NECA treatments. As these authors noted, paroxetine is membrane-permeant, and thus when specific binding is defined with unlabeled paroxetine, binding of [^{3}H]paroxetine obscures whether surface changes in SERT levels have occurred, particularly if surface SERT is a small fraction of the total. Consistent with this idea, we found no change in [^{125}I]RTI-55–labeled SERTs using paroxetine as the displacer and found that surface SERTs, as defined with unlabeled 5-HT, are only ~30% of the total transporter pool. Thus, if the 5-HT–sensitive binding is examined, the amount of this labeling is observed to increase upon NECA stimulation. Likewise, in A_3 AR/SERT cotransfected CHO cells, in which the amount of SERT protein expressed is sufficient to allow both surface binding and the use of biotinylation paradigms, we observed a receptor-mediated increase in both measures. The use of a ligand-independent biotinylation paradigm also further diminishes the possibility that NECA treatments leads to a conversion of low-affinity surface SERT molecules to sites that are both active and can bind antagonist.

Our findings indicate that A_3 ARs, via a PKG-linked pathway, alter SERT plasma-membrane density both in native preparations and in heterologous model systems. Such a conclusion is also supported by our studies with surface SERT inactivation by MTSET. If SERT increases after NECA stimulation are dependent solely on insertion of new carriers, the percentage increase should increase, assuming that no other pathways are modified by MTSET. Indeed, the NECA-induced increase of SERT activity on MTSET-treated cells increased substantially (~85%) compared with control conditions (~20%). Our observations of an enhanced stimulation would seem to preclude that increases arise from a reduced endocytosis of existing surface carriers.

Although A_3 AR stimulation leads to clear changes in SERT surface density in the two models we examined, we also gathered evidence for a change in transporter intrinsic activity. Here, activation of p38 MAPK seemed to be a critical determinant. p38 MAPK has been documented to play a role in catalytic activation of GLUT4 glucose transporters (Sweeney et al., 1999) and supports nontrafficking-dependent up-regulation of NET triggered by insulin (Apparsundaram et al., 2001). As with all inhibitor-based studies, the target of SB203580 and SB202190 could be something other than p38 MAPK (Godl et al., 2003). However, our immunoprecipitation studies indicate that p38 MAPK has constitutive activity in RBL-2H3 cells under our culture conditions, activity that can be further elevated by AR stimulation. The target for p38 MAPK in catalytic activation of SERT (or GLUT4) is as yet unknown. Intracellular SERTs that are being mobilized could be targeted by p38 MAPK, or alternatively, p38 MAPK may target newly inserted carriers. We do not yet know whether p38 MAPK targets SERT directly or supports catalytic activation through more indirect mechanisms. Activation of p38 MAPK has been linked to the membrane translocation and activation of PP2A (Liu and Hofmann, 2003). Although the locus of action of PP2A is probably multifactorial, it seems possible that p38 MAPK activation could thus stabilize plasma membrane SERT/PP2A complexes. On the other hand, activated PP2A could dephosphorylate SERT-associated proteins. Future studies will address these possibilities.

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