(predicted mass 2755, observed 2755), N,O6-diacetyl-MurNac-GlcNac pentapeptide (predicted mass 2828, observed 2826), murein-tetrapeptide-murein-pentapeptide (predicted mass 3990, observed 3995), (murein-tetrapeptide)$_2$-murein-pentapeptide (predicted mass 5194; observed 5196), and (murein-tetrapeptide)$_4$ (predicted mass 6285, observed 6286).

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22. Plasmid pGL4 contains the coding sequence of SEB-SPA$_{490-524}$, which was released from pSEB-SPA$_{490-524}$ by Eco RI–Bam HI digestion and inserted into the pT181 derivative pWil5. *S. aureus* SM317 (pGL4) was grown on TSB tet agar. A plasmid library of *S. aureus* OS2 chromosomal DNA was obtained by partial digestion with Sau 3A1. DNA fragments of 3 to 5 kb were purified and cloned into Bam HI–digested pC194-mcs, which contains the multiple cloning site of pUC19 inserted into the Hind III site of pC194. SM317 (pGL4) was transformed with the pC194-mcs plasmid library and transformants were selected on TSB tet-cm agar.
23. The DNA insertions of pGL1631 and 1834 were mapped and sequenced by synthesizing oligonucleotide primers. The primers for the amplification of *srtA* from the chromosomal DNA of *S. aureus* strains OS2 (pGL1897) and SM317 (pGL1898) were 5′-AAGGATCCAAAAGGAGCGGTATACATTGC-3′ and 5′-AAGGATCCTACCTTTTCCTCTAGCTGAAG-3′.
24. In another report we show that purified SrtA protein catalyzes the in vitro transpeptidation of substrates bearing an LPXTG motif (H. Ton-That, G. Liu, S. K. Mazmanian, K. F. Faull, O. Schneewind, in preparation).
25. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
26. We thank W. W. Navarre for help in determining the surface protein anchor structure of strain SM317, and D. Missiakas, P. Model, M. Russel, and members of our laboratory for critical reading of this manuscript. S.K.M. was supported by the Predoctoral Training Program in Genetic Mechanisms at UCLA (T32GM07104). H.T.-T. was supported by the Microbial Pathogenesis Training Grant at UCLA (AI 07323). Work in O.S.'s laboratory is supported by grant AI33987 from the National Institute of Allergy and Infectious Diseases.

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Phosphorylation and Sequestration of Serotonin Transporters Differentially Modulated by Psychostimulants

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Many psychotropic drugs interfere with the reuptake of dopamine, norepinephrine, and serotonin. Transport capacity is regulated by kinase-linked pathways, particularly those involving protein kinase C (PKC), resulting in transporter phosphorylation and sequestration. Phosphorylation and sequestration of the serotonin transporter (SERT) were substantially impacted by ligand occupancy. Ligands that can permeate the transporter, such as serotonin or the amphetamines, prevented PKC-dependent SERT phosphorylation. Nontransported SERT antagonists such as cocaine and antidepressants were permissive for SERT phosphorylation but blocked serotonin effects. PKC-dependent SERT sequestration was also blocked by serotonin. These findings reveal activity-dependent modulation of neurotransmitter reuptake and identify previously unknown consequences of amphetamine, cocaine, and antidepressant action.

Serotonin [5-hydroxytryptamine (5-HT)] is a platelet-stored vasoconstrictor that also acts as a transmitter in the nervous system to modulate a wide spectrum of behaviors (*1*). The actions of 5-HT are terminated by active transport (*2*). Whereas 5-HT actions are mediated by >15 different types of receptors, a single 5-HT transporter (SERT) is responsible for extracellular 5-HT clearance (*3*). SERT activity is blocked by cocaine and tricyclic antidepressants. Serotonin-selective reuptake inhibitors (SSRIs) like fluoxetine (Prozac) preferentially block SERTs and enhance serotonergic signaling in affective disorders (*2, 4*). The amphetamines are substrates for SERTs, as well as for dopamine (DA) and norepinephrine (NE) transporters (DATs and NETs, respectively) (*5*) and can trigger SERT-mediated release of 5-HT (*5–8*). Repeated administration of amphetamines sensitizes monoaminergic synapses to subsequent psychostimulant challenge (*9*), which may involve modulated protein kinase cascades (*10*). Alterations in SERT activity and binding site density (*11*) and SERT gene polymorphisms (*12*) have implicated the transporter in anxiety, depression, suicide, autism, and substance abuse. Recent findings with transgenic mice (*13*) support an important role for 5-HT and SERTs in the behavioral actions of cocaine and amphetamine.

SERT expression can be rapidly modulated by receptor stimulation, second messenger production, and kinase activation (*14–16*). Suppression of SERT activity accompanying protein kinase C (PKC) activation (*17*) arises from a loss of 5-HT uptake capacity (V_{max}). The loss in 5-HT uptake capacity correlates with a loss of surface-expressed SERTs (*17*), similar to the PKC modulation of homologous γ-aminobutyric acid (GABA), DA, and NE transporters (*18*). PKC activators and phosphatase inhibitors induce SERT phosphorylation (*19*) with a similar time course and kinase antagonist sensitivity as observed for changes in 5-HT transport.

We investigated whether the regulation of SERTs was influenced by transport and whether SERT ligands differentially influenced SERT regulation. Figure 1A shows that PKC-mediated SERT phosphorylation in transfected human embryonic kidney–293 (HEK-293) cells was substantially diminished if assayed in the presence of the transported neurotransmitter, 5-HT. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of immunoprecipitates from [^{32}P]PO_4-labeled cell extracts (*20*) revealed a three- to fivefold stimulation of human SERT (hSERT) phosphorylation after phorbol 12-myristate 13-acetate (β-PMA) application. This stimulation was abolished by coapplication of PKC antagonists. In the presence of 5-HT (1 μM), SERT phosphorylation triggered by phorbol esters was also substantially blunted. At low concentrations of β-PMA (for example, 10 nM), 5-HT essentially abolished phorbol ester–induced SERT labeling, At 200 nM β-PMA, where labeling of SERTs is maximal, we consistently achieved 40 to 60% inhibition of SERT phosphorylation at maximal concentrations of 5-HT (1 μM) with a median effective concentration (EC50) of 70 nM (Fig. 1B).

If the actions of 5-HT on SERT phosphorylation arise as a consequence of transport, then an intrinsic homeostatic loop might be present to link transporter expression to extracellular amine availability, and coincubation with SERT antagonists should block this effect. Indeed, the SERT-selective tricyclic antidepressant imipramine (1 μM) or the SSRIs paroxetine (1 μM) and citalopram (1 μM) blocked the ability of 5-HT to limit PKC-dependent SERT phosphorylation (Fig. 1C). This effect was selective for SERT antagonists, as neither the DAT inhibitor GBR-12909 nor the NET antagonist nisoxetine could affect the ability of 5-HT to blunt SERT phosphorylation (Fig. 1C). There are no known 5-HT receptor subtypes on HEK-293 cells, and to our knowledge, 5-HT does not induce acute changes in cyclic adenosine 5′-monophosphate, inositol trisphosphate, or intracellular Ca^{2+} levels in these cells. Moreover, the ability of 5-HT to diminish PKC-dependent SERT phosphorylation was not

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affected by coincubation with the 5-HT receptor antagonists mesulergine, clozapine, or ketanserin (Fig. 1D). Thus, it is unlikely that 5-HT receptors are involved in the actions of 5-HT to blunt SERT phosphorylation. These results suggest that intrinsic SERT activity governs the transporter's phosphorylation.

We evaluated whether the activity dependence of SERT phosphorylation was revealed by other SERT substrates. The SERT substrates D-amphetamine and fenfluramine (*5–8, 21*) reduced SERT phosphorylation to a similar extent as 5-HT (Fig. 2A). Unlike 5-HT or amphetamines, DA and NE are poor substrates for SERTs and were found to be ineffective at modulating SERT phosphorylation at concentrations where 5-HT substantially reduces PKC-mediated SERT labeling (Fig. 2A).

Substrate permeation through SERTs requires extracellular Na^+ and Cl^- (Fig. 2B) (*3, 22*). Individual substitution for Na^+ or Cl^- partially reversed the ability of 5-HT to diminish SERT phosphorylation, and an almost complete reversal was achieved when both ions were substituted together. Substitution for these ions had no effect on SERT phosphorylation in the absence of 5-HT (*23*). The Michaelis constant K_m for 5-HT transport is substantially higher than the EC50 for 5-HT suppression of PKC-mediated SERT phosphorylation (Fig. 1B), suggesting that phosphorylation may be closely linked to high-affinity steps in the translocation cycle, such as initial substrate binding (*6, 24*). Importantly, 5-HT–preloading experiments (Fig. 2C) revealed that the suppression of phosphorylation was not a consequence of an increase in intracellular 5-HT (*20*). We also found no evidence that 5-HT application alters cellular PKC activity (*25*). Rather, suppression of SERT phosphorylation requires transport activation that is coincident with PKC activation.

PKCs are not the only protein kinases that phosphorylate SERTs in HEK-293 cells (*19*). The SERT phosphorylation that arises from PKA activation by cholera toxin is insensitive to PKC antagonists, as is most of the phosphorylation achieved after protein phosphatase 2A inhibition with okadaic acid. 5-HT had no influence on the phosphorylation status of SERT triggered by cholera toxin (Fig. 2D), and phosphorylation of SERT arising from okadaic acid treatment was diminished only to a level achieved with PKC inhibitors [see table 2 of (*19*)], consistent with the unmasking of other cellular kinases that target SERT in a 5-HT–independent manner.

Activation of PKC results in a loss of transport capacity, sequestration of transporter proteins, or phosphorylation of multiple members of the Na^+/Cl^- coupled neurotransmitter transporter gene family (or all of these effects) (*15, 17, 18, 26–30*). If phosphorylation after PKC activation participates in transporter sequestration, then the loss of phorbol ester–triggered phosphorylation of SERT should also diminish the effects of phorbol esters on SERT trafficking. We thus biotinylated surface SERTs (*31*) to explore the impact of extracellular substrate on transporter surface expression. Treatment with β-PMA (1 μM) for 30 min induced a 30 to 40% reduction in surface pools of SERT protein and a concomitant increase in nonbiotinylated intracellular SERT protein, indicative of transporter sequestration (Fig. 3A). 5-HT was able to prevent alterations in SERT surface expression achieved with β-PMA. Critical domains for PKC-mediated phosphorylation and sequestration may be differentially exposed during substrate translocation. GAT1 GABA transporters are less sensitive to trypsin proteolysis if GABA is present (*32*), implying altered transporter structure upon substrate binding or translocation. DATs and SERTs display altered accessibility to inactivating methane thiosulfonate reagents after ligand occupancy (*33*). Alternatively, the activities or localization of an associated kinase or phosphatase (*34*) could be altered by transport (Fig. 3B). Phosphorylation suppression might also rely on the excess ion flow that occurs during 5-HT translocation (*35*). Because PKC-dependent SERT regulation occurs under voltage clamp (*17*) and β-PMA–triggered SERT phosphorylation is insensitive to 30 min of ouabain treatment (1 mM) (*23*), it is unlikely that the effects we report are indirect consequences of membrane depolarization or a rundown of ion gradients.

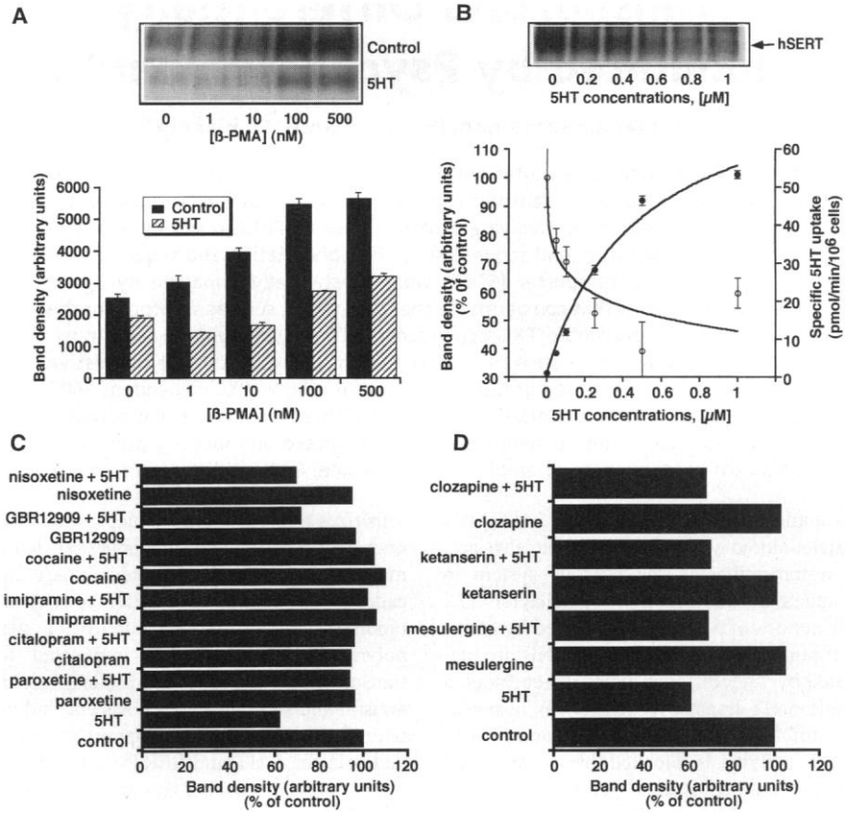

Fig. 1. 5-HT modulation of phorbol ester–triggered hSERT phosphorylation in stably transfected HEK-293 cells. (**A**) Effect of coincubation of 5-HT (1 μM) on dose-dependent phosphorylation of SERT as revealed by immunoprecipitation (*20*), SDS-PAGE, and autoradiography (top panel). The bottom panel presents the average band intensity from three experiments. Error bars indicate SEM. (**B**) SERT phosphorylation induced by β-PMA (200 nM) for 30 min was evaluated as in (A), using different concentrations of 5-HT (top and bottom panels). hSERT activity (*38*) was assessed as a function of increasing concentrations of 5-HT in the presence of 200 nM β-PMA (bottom panel). Error bars indicate SEM. The EC50 for the 5-HT block of hSERT phosphorylation was 68 ± 21 nM. The K_m for 5-HT transport was 495 ± 60 nM. Results are mean values from three experiments. The effect of 5-HT on SERT phosphorylation at 10 to 500 nM β-PMA was statistically significant ($P < 0.05$, Student's two-tailed t test). (**C**) Sensitivity of the 5-HT–mediated reduction in hSERT phosphorylation to amine transporter antagonists (1 μM). (**D**) Lack of sensitivity of the 5-HT–mediated reduction in hSERT phosphorylation to 5-HT receptor antagonists (50 μM). For (C) and (D), antagonists were added 20 min before treatment with β-PMA (200 nM) for 30 min or coapplication of β-PMA/5-HT (1 μM). Experiments presented in (C) and (D) were repeated with essentially equivalent results.

Control of transporter surface expression by external substrates represents a novel homeostatic mechanism that may serve in the neuron to fine-tune transport capacity to match demands imposed by fluctuating levels of neurotransmitter. Because extracellular GABA can impact GAT1 trafficking (*36*), this regulatory mechanism may be of general relevance for other transporters. Amphetamines substitute for 5-HT in suppressing PKC-mediated SERT phosphorylation. Such action could override homeostatic transporter sequestration processes and provide for psychostimulant sensitization by increasing the number of psychostimulant targets available to a subsequent stimulus. Given the homology among SERTs, NETs, and DATs, it is possible that altered trafficking of amine transporters may represent one of many molecular changes underlying psychostimulant sensitization and withdrawal mechanisms

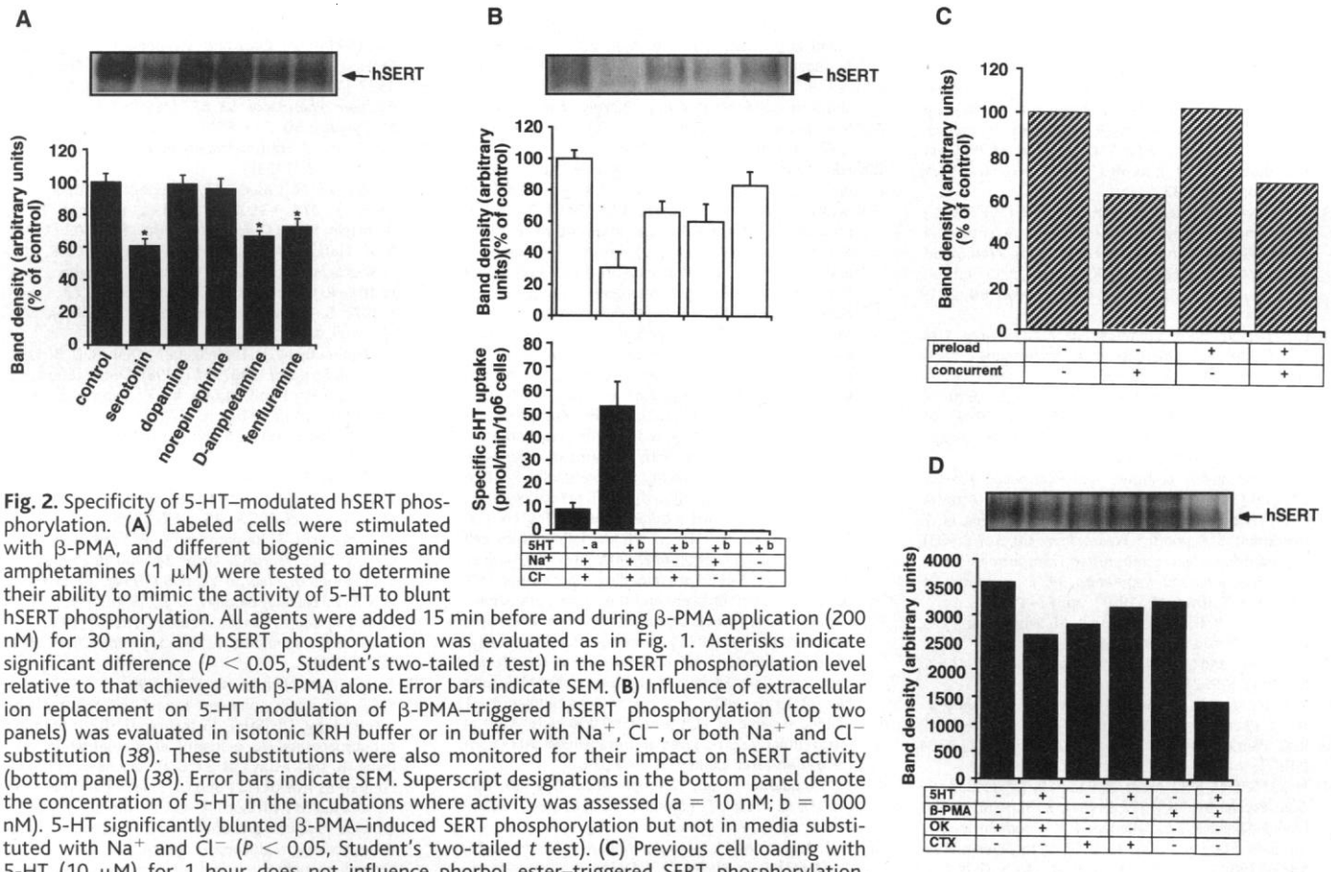

Fig. 2. Specificity of 5-HT–modulated hSERT phosphorylation. (**A**) Labeled cells were stimulated with β-PMA, and different biogenic amines and amphetamines (1 μM) were tested to determine their ability to mimic the activity of 5-HT to blunt hSERT phosphorylation. All agents were added 15 min before and during β-PMA application (200 nM) for 30 min, and hSERT phosphorylation was evaluated as in Fig. 1. Asterisks indicate significant difference ($P < 0.05$, Student's two-tailed t test) in the hSERT phosphorylation level relative to that achieved with β-PMA alone. Error bars indicate SEM. (**B**) Influence of extracellular ion replacement on 5-HT modulation of β-PMA–triggered hSERT phosphorylation (top two panels) was evaluated in isotonic KRH buffer or in buffer with Na^+, Cl^-, or both Na^+ and Cl^- substitution (*38*). These substitutions were also monitored for their impact on hSERT activity (bottom panel) (*38*). Error bars indicate SEM. Superscript designations in the bottom panel denote the concentration of 5-HT in the incubations where activity was assessed (a = 10 nM; b = 1000 nM). 5-HT significantly blunted β-PMA–induced SERT phosphorylation but not in media substituted with Na^+ and Cl^- ($P < 0.05$, Student's two-tailed t test). (**C**) Previous cell loading with 5-HT (10 μM) for 1 hour does not influence phorbol ester–triggered SERT phosphorylation, whereas concurrent 5-HT application (1 μM) blunts labeling. (**D**) 5-HT (1 μM) selectively impacts PKC-dependent SERT phosphorylation. Agents tested were β-PMA (200 nM), cholera toxin (1 ng/ml), or okadaic acid (1 μM), all for 30 min. Experiments presented in (C) and (D) were repeated with essentially equivalent results.

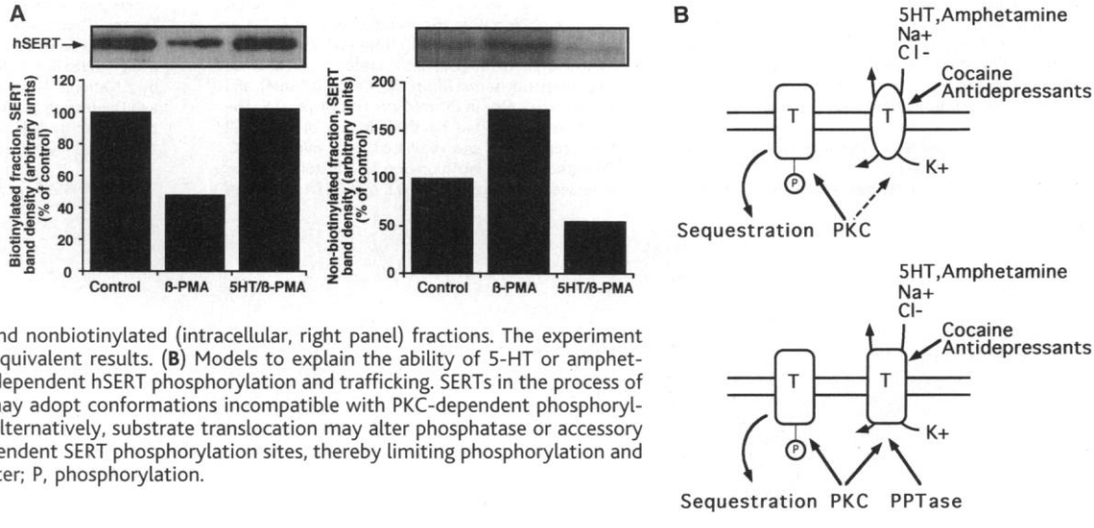

Fig. 3. Functional impact of 5-HT–modulated hSERT phosphorylation. (**A**) The ability of 5-HT to modulate hSERT sequestra-tion was examined through cell surface biotinylation (*31*). Cells were treated for 30 min with either β-PMA (1 μM) or β-PMA and 5-HT (1 μM) before biotinylation and immunoblotting of biotinylated (cell surface, left panel) and nonbiotinylated (intracellular, right panel) fractions. The experiment was repeated once with equivalent results. (**B**) Models to explain the ability of 5-HT or amphetamines to modulate PKC-dependent hSERT phosphorylation and trafficking. SERTs in the process of translocating substrates may adopt conformations incompatible with PKC-dependent phosphorylation and sequestration. Alternatively, substrate translocation may alter phosphatase or accessory protein access to PKC-dependent SERT phosphorylation sites, thereby limiting phosphorylation and sequestration. T, transporter; P, phosphorylation.

(*37*). Nonpermeant ligands like the SSRIs that prevent 5-HT permeation may have therapeutic utility in disease states, not only by preventing 5-HT reuptake but also by allowing kinase-linked signaling pathways to shift the cellular distribution of SERTs.

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20. HEK-293 cells stably expressing hSERT (293-hSERT) were grown, passaged, in vivo labeled with [^{32}P] orthophosphate, and subjected to SERT (CT-2B) immunoprecipitation/SDS-PAGE and autoradiography as previously described (*19*). Protein immunoblots and immunoprecipitation experiments demonstrate that hSERT protein migrates largely as a single broadband of 96 kD (*19*). For experiments involving 5-HT modulation of SERT phosphorylation, 5-HT was preincubated for 20 min before and then during β-PMA, cholera toxin, or okadaic acid application. In experiments testing the role of Na^+ and Cl^-, the labeled cells were washed multiple times with Krebs-Ringers-Hepes (KRH) buffer that is free from Na^+ and Cl^- (*19*) and then incubated with prewarmed buffer, with or without 5-HT. The ability of intracellular 5-HT to influence β-PMA–stimulated hSERT phosphorylation was assessed in KRH media by preloading cells at 37°C in 10 μM 5-HT for 1 hour, followed by cell washing and application of β-PMA (200 nM), with or without 1 μM 5-HT. For preloading experiments, cells were washed extensively and then lysed, and supernatants were recovered for high-performance lipid chromatography (HPLC) determination of steady-state intracellular 5-HT accumulation in the Neurochemistry Core Facility of the Center for Molecular Neuroscience. HPLC analysis of intracellular 5-HT yielded a value of 200 pmol/500,000 cells. A 10-pl intracellular volume gives an intracellular 5-HT concentration after loading of ~40 μM.
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