Antidepressant-sensitive serotonin (5-hydroxytryptamine, 5HT) transporters (SERTs) are responsible for efficient synaptic clearance of extracellular 5HT. Previously (Qian, Y., Galli, A., Ramamoorthy, S., Risso, S., DeFelice, L. J., and Blakely, R. D. (1997) J. Neurosci. 17, 45–47), we demonstrated that protein kinase (PKC)-linked pathways in transfected HEK-293 cells lead to the internalization of cell-surface human (h) SERT protein and a reduction in 5HT uptake capacity. In the present study, we report that PKC activators rapidly, and in a concentration-dependent manner, elevate the basal level of hSERT phosphorylation 5–6-fold. Similarly, protein phosphatase (PP1/PP2A) inhibitors down-regulate 5HT transport and significantly elevate hSERT 32P incorporation, effects that are additive with those of PKC activators. Moreover, hSERT phosphorylation induced by β-phorbol 12-myristate 13-acetate is abolished selectively by the PKC inhibitors staurosporine and bisindolylmaleimide I, whereas hSERT phosphorylation induced by phosphatase inhibitors is insensitive to these agents at comparable concentrations. Protein kinase A and protein kinase G activators fail to acutely down-regulate 5HT uptake but significantly enhance hSERT phosphorylation. Basal hSERT and okadaic acid-induced phosphorylation were insensitive to chelation of intracellular calcium and Ca2+/calmodulin-dependent protein kinase inhibitors. Together these results reveal hSERT to be a phosphoprotein whose phosphorylation state is likely to be tightly controlled by multiple kinase and phosphatase pathways that may also influence the transporter’s regulated trafficking.

The biogenic amine, serotonin (5-hydroxytryptamine, 5HT),1

1 The abbreviations used are: 5HT, 5-hydroxytryptamine; SERT, serotonin transporter; DAT, dopamine transporter; NÉ, norepinephrine; NET, norepinephrine transporter; PKC, protein kinase C; PKA, protein kinase A; PKG, protein kinase G; CaM kinase, Ca2+/calmodulin-dependent kinase; PP1/2A, protein phosphatase 1/2A; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; BAPTA-AM, [1,2-bis(o-aminophenoxyl)ethane-N,N’-tetraacetate] tetraacetoxymethyl ester; KN-93, [2-[(N-methyl-L)-tyrosyl]-4-phenylpiperazine]; KN-93, [2-(N(2-hydroxyethyl)-N-(4-methoxybenzenesulfanyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine]; h, human; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; DMEM, Dulbecco’s modified Eagle’s medium.

This work was supported by National Institutes of Health Grant DA07390 (to R. D. B.) and a National Alliance for Research on Schizophrenia and Depression Young Investigator Award (to S. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Phosphorylation and Regulation of Antidepressant-sensitive Serotonin Transporters*

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uptake may occur, in part, as a consequence of transporter phosphorylation. Direct protein phosphorylation is known to regulate the activity and/or surface distribution of many ion channels, receptors, and transporters; however, to date, SERT phosphorylation has not been described.

To explore regulatory posttranslational processing of SERT proteins, we have generated and characterized SERT-specific antibodies that immunoprecipitate and immunoblot SERT polypeptides in vitro and in vivo (1, 36). We now report the use of these antibodies to establish the direct phosphorylation of hSERT proteins using 293-hSERT cells. hSERT proteins in this system are phosphorylated under basal conditions, and phosphorylation can be significantly elevated by both PKC and cyclic nucleotide (cAMP and cGMP)-activated protein kinases. In addition, studies with phosphatase inhibitors reveal endogenous pathways leading to SERT phosphorylation independent of PKC, PAK, and PKG. These findings reveal a highly dynamic, and potentially complex, process of SERT phosphorylation and dephosphorylation whose regulation coincides, in part, with altered trafficking of SERT proteins. We propose that direct SERT phosphorylation may be a determinant of receptor and second messenger-mediated changes in 5HT transport capacity and discuss the potential roles of SERT phosphorylation in altered plasma membrane expression.

EXPERIMENTAL PROCEDURES

Materials—293-hSERT cells were previously generated and characterized in this laboratory (1). Trypsin, glutamine, penicillin, streptomycin, G418, and phosphate-free Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Life Technologies, Inc., or obtained from the Vanderbilt Media Care. Cys/Met-free DMEM was obtained from Cellgro. PMA and phorbol 12,13-dibutyrate isomers, staurosporine, chelation toxin, dialyzed fetal bovine serum, and protease inhibitors were obtained from Sigma. Okadaic acid, KT5720, KN-62, KN-93, 8-(4-chlorophenylthio)guanosine 3′,5′-cyclic monophosphate, 8-(4-chlorophenylthio)guanosine 3′,5′-cyclic monophosphate were purchased from LC laboratories/Alexis Biochemicals. Bisindolylmaleimide I, calycin A, cyclosporin A, (−)-indolactam V, BAPTA-AM, and microcinostatin were purchased from Calbiochem. [3H]5-HT (5-hydroxy-[3H]tryptamine tri-thio)guanosine 3′,5′-cyclic monophosphate, 8-(4-chlorophenylthio)guanosine 3′,5′-cyclic monophosphate were purchased from LC laboratories/Alexis Biochemicals. BAPTA-AM (100 μmol/ml) was obtained from ICN Biotech Inc. All reagents were of the highest grade possible from standard commercial sources.

Cell Culture—293-hSERT and parental HEK-293 lines were maintained in monolayer culture in 75-cm² flasks in an atmosphere of 5% CO₂ at 37 °C as described previously (1). Both lines were grown in DMEM supplemented with 4% dialyzed fetal bovine serum, 2 μg/ml glatiramer, 100 units/ml penicillin, and 100 μg/ml streptomycin. Medium for the transfected line was supplemented with G418 (250 μg/ml). Use of dialyzed serum at a 1000 molecular weight cutoff was necessary to prevent loss of expression of 5HT uptake and SERT protein expression through, as yet, undefined mechanisms.

Assay of 5-HT Transport—[3H]5-HT transport activity was assayed in monolayer cultures for the times indicated at 37 °C as described previously (1). Briefly, cells were plated on poly-L-lysine (0.1 mg/ml)-coated 6-well (500,000 cells/well) or 24-well (100,000 cells/well) plates 48 h before experiments. At assay, the medium was removed by aspiration, and the cells were washed with 2 ml of Krebs-Ringer’s (KR) buffer containing 130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 1.8 g/liter glucose, 10 HEPES, pH 7.4. Cells were then incubated in KRH buffer containing 100 μM pargline and 100 μM ascorbic acid for 10 min at 37 °C with or without various modulators. 5HT uptake assays were initiated by the addition of [3H]5-HT (1 μM final concentration), and the assays were terminated by three rapid washes (2 ml each) with KRH buffer at room temperature containing 100 μM imipramine. Cells were then solubilized in 1% SDS or Optiphase prelabeled scintillation mixture (Wallac, Gaithersburg, MD), and [3H]5-HT accumulation was determined by liquid scintillation spectrometry. Specific 5HT uptake was determined by subtracting the amount accumulated [3H]5-HT in the presence of 1 μM paroxetine. Statistical analyses comparing vehicle and modulator-modified uptake were performed using Student’s paired t tests.

Metabolic Labeling and Immunoprecipitations—For phosphorylation studies, 293-hSERT cells were seeded on poly-n-lysine-coated 6-well plates at 5 × 10⁵ cells/well. After 48 h, monolayers were washed once in phosphate-free DMEM and incubated for 1 h at 37 °C. Typically, cells were then incubated at 37 °C with the same medium containing 1 mM CaCl₂/100 μM carrier-free [32P]orthophosphate for 1 h to equilibrate the intracellular level of orthophosphate. After incubation, the adherent cells were washed three times with phosphate-buffered saline and lysed by the addition of 400 μl/well ice-cold modified radioimmunoprecipitation (RIPA, 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, pH 7.4) buffer containing protease (1 μg peptatin A, 250 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprolin) and phosphatase inhibitors (10 mM sodium fluoride, 50 mM sodium pyrophosphate, and 1 μM okadaic acid) for 1 h at 4 °C with agitation. RIPA extracts were centrifuged at 20,000 × g for 30 min at 4 °C. Protein content of supernatant was assessed using the DC protein assay (Bio-Rad) with bovine serum albumin as the standard. Protein content between wells and experiments showed <5% variability. Labeling with Trans32P-label was carried out in Cys/ Met-free DMEM, as described previously (36, 37). Supernatants were preclarified by the addition of 100 μl (3 mg) of Protein A-Sepharose beads for 1 h at 4 °C. hSERT protein was immunoprecipitated overnight at 4 °C by the addition of SERT-specific antibody, CT-2 (10 μl of antiserum) on end-over-end continuous mixing, followed by 1-h incubation with Protein A-Sepharose beads (3 mg in 100 μl in RIPA buffer) at 22 °C. Additional experiments to test specificity were carried out with the non-SERT-specific antibody N430 (37), CT-2B preimmune serum, or a second SERT-specific serum S365 (36). The immunoadsorters were washed three times with ice-cold RIPA buffer prior to the addition to 50 μl of Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.1% bromphenol blue), incubated for 30 min at 22 °C, and then resolved by SDS-PAGE (10%), with radiolabeled proteins detected by autoradiography or direct PhosphorImager (Molecular Dynamics) analysis. Quantification of the relative amounts of 32P incorporated into hSERT protein were estimated using ImageQuant software (Molecular Dynamics). Quanti"cation from digitized autoradiograms was evaluated on multiple film exposures to ensure quantitation within the linear range of the film and gave identical results to estimations achieved with direct PhosphorImager quantitation.

RESULTS

Immunoprecipitation of Phosphorylated hSERT Protein—To determine whether hSERT proteins are subject to phosphorylation, we metabolically labeled stably transfected 293-hSERT cells with [32P]orthophosphate and immunoprecipitated determinant extracts with a set of SERT-specific and control antisera. In these initial experiments, the Ser/Thr phosphatase inhibitor okadaic acid (1 μM) was applied to intact cells before extraction in an attempt to preserve labeling from endogenous kinases. A more systematic analysis of the effects of okadaic acid-induced labeling is presented later in this report. SDS-PAGE/autoradiography of immunoprecipitates from labeled 293-hSERT cells reveals a broad band centered at ~96 kDa (Fig. 1), the size expected from immunobots for mature, N-glycosylated hSERT protein (1, 36). The 96-kDa band is absent from immunoprecipitates of parental HEK-293 cells, metabolically labeled, and extracted in parallel. The 96-kDa band is also not immunoprecipitated from transfected cell extracts if CT-2 preimmune serum, the NET-specific antibody N430, or CT-2 serum preabsorbed with CT-2/GST fusion protein are utilized for immunoprecipitations. The 96-kDa band is retained, however, if CT-2 antiserum is preabsorbed with GST, the protein carrier for the fusion protein utilized to raise the CT-2 antibody. The SERT antipeptide antibody S365 (36), like CT-2 antiserum, immunoprecipitates the same 96-kDa band, although the S365 antipeptide antibody displays consistently lower recovery in
the presence of 1 M-PMA and 1 μM okadaic acid. RIPA extraction, immunoprecipitation, SDS-PAGE, and autoradiography were performed as described previously (and in subsequent figures) with okadaic acid treatment but is nonetheless readily apparent when compared to nontransfected HEK-293 cells. This basal level of labeling is certainly lower than described previously (36). An autoradiogram of a 10% SDS-PAGE is shown which is representative of three experiments. The location of protein molecular mass standards in kDa electrophoresed in parallel is shown to the left of the figure. The position marked for 32P-labeled hSERT (~96 kDa, arrow) matches that observed with direct immunoblotting. An asterisk denotes the position of a labeled coprecipitating product believed to be a degradation product or lightly glycosylated form of SERT protein.

Both 32P- and 35S-metabolic labeling paradigms (data not shown). In addition, we have raised another two polyclonal antisera against the COOH terminus of SERT and found that, like CT-2 and S-365 sera, they also immunoprecipitate a phosphorylated 96-kDa band selectively from 293-hSERT cell extracts (data not shown). Together these findings support the contention that the phosphorylated 96-kDa species immunoprecipitated from 293-hSERT extracts represents posttranslationally modified hSERT protein. CT-2 antisera also immunoprecipitates a minor species at ~76 kDa (Fig. 1, *) that we suspect is a less heavily glycosylated or partially degraded form of hSERT protein. Together, these data suggest that hSERT protein is a target for direct phosphorylation by endogenous protein kinases and phosphatases in stably transfected HEK-293 cells.

Stimulation of hSERT Phosphorylation by Activation of PKC—293-hSERT cells display PKC-dependent down-regulation of 5HT uptake capacity and hSERT-associated currents associated with a reduction in surface transporter protein (1). We tested whether PKC activation affects the phosphorylation state of hSERT protein. In the absence of phosphatase inhibitors or PKC activators (Fig. 2, 0 min time point), we immunoprecipitated phosphorylated hSERT protein from 293-hSERT cells. This basal level of labeling is certainly lower than described previously (and in subsequent figures) with okadaic acid treatment but is nonetheless readily apparent when compared with immunoprecipitations from nontransfected HEK-293 cells that are electrophoresed in parallel. Treatment of transfected cells with the PKC activator β-PMA induces a time- and concentration-dependent augmentation of basal hSERT phosphorylation at maximal time and concentration points. The effects of β-PMA are rapid, as 1 μM β-PMA increases hSERT phosphorylation by more than 2-fold within the first 5 min of application (Fig. 2). We repeated our published analyses of β-PMA-induced inhibition of 5HT uptake so that transport and phosphorylation analyses could be followed in parallel, and we can demonstrate that the elevation in basal hSERT phosphorylation induced by β-PMA displays a similar time course to the β-PMA-induced losses in 5HT transport (Fig. 2C). As with phosphorylation, the rate of change in uptake is greatest after the first 5 min of β-PMA treatment and then rises gradually thereafter to essentially plateau by 60 min. Low concentrations of β-PMA were required to effect an increase in hSERT phosphorylation.

Both hSERT phosphorylation and 5HT uptake inhibition are observed at low concentrations of β-PMA (Fig. 3, A and C). A greater than 2-fold increase in phosphorylation is evident with 1 nM β-PMA (60-min assay), and the EC50 for phosphorylation was 1.5 nM. A similar concentration dependent in β-PMA regulation of [3H]5HT uptake was observed with reductions in 5HT transport observed with 1 nM β-PMA. Like β-PMA, the PKC activators, β-PDBu and indolactam V, stimulated 32P incorporation into hSERT protein 4–6-fold (Fig. 4). Moreover,

![Fig. 1. Phosphorylation of hSERT in 293-hSERT cells.](http://example.com/fig1.png)

![Fig. 2. Time-course effect of β-PMA on phosphorylation of hSERT and 5-HT uptake.](http://example.com/fig2.png)
...of brine (200 nM) and bisindolylmaleimide I (1
capturing basal PKC activity or reflect labeling of hSERT by distinct
kinases. Since okadaic acid might be stabilizing the effects
of okadaic acid-stimulated hSERT phosphorylation. Like PKC-mediated phosphorylation of hSERT, incorporation of
$^{32}$P into hSERT following okadaic acid (1 µM) treatment is rapid, with a nearly 3-fold increase over basal phosphorylation achieved within 5 min (Fig. 5, A and B) and a gradual increase evident to 1 h of treatment. However, unlike hSERT phosphorylation following β-PMA treatment, a pronounced rise in SERT phosphorylation is evident between 60 and 120 min after okadaic acid treatment. Like β-PMA, okadaic acid treatment reduces 5HT uptake in 293-hSERT cells, although the time course of the response is significantly distinct from that of phorbol esters (Fig. 5C). As with okadaic acid-stimulated hSERT phosphorylation, we observe the appearance of enhanced down-regulation of [H]5HT uptake from 60 to 120 min of treatment. Concentration-response studies with okadaic acid (Fig. 6) reveal maximal effects (6–9-fold) observed by 1 µM (Fig. 5) and an EC$_{50}$ for hSERT phosphorylation of ~350 nM, similar to that found for the concentration dependence of okadaic acid-triggered reductions in 5HT uptake (Fig. 6C). We also found the potent PP1/2A inhibitor calyculin A to augment hSERT phosphorylation to a similar extent as okadaic acid, but we found the PP2B inhibitor microcystin to be relatively ineffective (Table I).

Next we asked whether β-PMA-stimulated and okadaic acid-induced hSERT phosphorylation act through a common mechanism by coapplication studies and cross-inhibitor studies. As shown in Fig. 7, β-PMA and okadaic acid each at maximally efficacious concentrations yield additive hSERT phosphorylation when coapplied, achieving more than a 10-fold increase in $^{32}$P incorporation over basal conditions in these experiments. Note again that okadaic acid achieves an increase in hSERT phosphorylation between 60 and 120 min that is not observed with β-PMA. These findings suggest that PKC and okadaic acid-inhibitable phosphatases in HEK cells phosphorylate hSERT through distinct pathways. Further evidence in support of this contention was gathered when we tested the ability...
PKC inhibitors to block okadaic acid-induced phosphorylation (Table I). Staurosporine and bisindolylmaleimide I at concentrations that fully block the β-PMA triggered phosphorylation of hSERT (refer to Fig. 4) fail to alter significantly okadaic acid-induced hSERT phosphorylation. Phosphorylation of hSERT revealed by okadaic acid treatment was also insensitive to intracellular calcium chelation as well as inhibition of CaM kinase II by KN-93 and KN-62 (Table I).

PKA-mediated hSERT Phosphorylation Is Distinct from PKC and Phosphatase Pathways—Differences in the time course and inhibitor sensitivity of β-PMA- and okadaic acid-induced hSERT phosphorylations suggest the involvement of one or more kinases distinct from PKC. Purified cytoplasmic NH₂ and COOH termini of rSERT are substrates for PKA as well as PKC in vitro (17, 35). We found the PKA activators forskolin and cholera toxin elevate hSERT phosphorylation 4–5-fold (Fig. 8). A similar level of hSERT phosphorylation is achieved with the membrane-permeant cAMP analog, 8-pCT-cAMP. The effect of cholera toxin is dose- and time-dependent with significant effects (3-fold) on incorporation of ³²P in hSERT observed at 10 ng/ml (data not shown). In time course studies, the cholera toxin-induced phosphorylation of hSERT is more delayed and gradual than observed for PKC activators, with a doubling of hSERT labeling not observed for 45 min and another doubling evident by 90 min of incubation (data not shown). Furthermore, unlike β-PMA and okadaic acid, PKA activators did not alter 5HT transport levels (data not shown). The PKC inhibitors staurosporine and bisindolylmaleimide I, at concentrations that block β-PMA effects on hSERT phosphorylation (Fig. 4), fail to reduce cholera toxin and forskolin-triggered phosphorylation (Fig. 8). However, the specific PKA inhibitor KT5720, which does not block the β-PMA-triggered labeling of hSERT (Fig. 4), blocks the effects of PKA activators completely (Fig. 8). Consistent with the inability of PKA inhibitors to mimic the effects of PKC inhibitors or attenuate the effects of okadaic acid.
TABLE I

Effect of phosphoprotein phosphatase inhibitors and its kinase selectivity on hSERT phosphorylation

293-hSERT cells were metabolically labeled with [32P]orthophosphate and incubated at 37 °C for 1 h in the absence (none) and presence of the following agents: okadaic acid, 1 μM; calyculin A, 1 μM; microcystin-LR, 5 μM; cyclosporin A, 5 μM; staurosporine, 200 nM; bisindoylmaleimide I, 500 nM; KT5720, 1 μM; KN-62, 2 μM; KN-93, 2 μM, and BAPTA-AM, 10 μM. Protein kinase inhibitors (staurosporine, bisindoylmaleimide I, KT5720, KN-62, KN-93, and BAPTA-AM) were added 30 min prior to the addition of okadaic acid. RIPA extraction, immunoprecipitation, SDS-PAGE, and autoradiography were performed as described under “Experimental Procedures.” Autoradiograms from three different experiments were scanned densitometrically, and the mean values ± S.E. were given. Statistical significance was calculated comparing treated to untreated samples using the paired Student’s t test.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Band density (arbitrary units)</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1192 ± 121</td>
<td>100 ± 10</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>5046 ± 236</td>
<td>423 ± 20*</td>
</tr>
<tr>
<td>Calyculin A</td>
<td>4982 ± 241</td>
<td>415 ± 19*</td>
</tr>
<tr>
<td>Microcystin</td>
<td>2458 ± 199</td>
<td>206 ± 17*</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>1381 ± 112</td>
<td>116 ± 19</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>1025 ± 199</td>
<td>86 ± 16</td>
</tr>
<tr>
<td>Okadaic acid + staurosporine</td>
<td>4817 ± 209</td>
<td>404 ± 18*</td>
</tr>
<tr>
<td>Bisindoylmaleimide I</td>
<td>1191 ± 125</td>
<td>100 ± 11</td>
</tr>
<tr>
<td>Okadaic acid + Bisindoylmaleimide I</td>
<td>4680 ± 355</td>
<td>393 ± 30*</td>
</tr>
<tr>
<td>KT5720</td>
<td>1185 ± 127</td>
<td>100 ± 11</td>
</tr>
<tr>
<td>Okadaic acid + KT5720</td>
<td>4492 ± 366</td>
<td>377 ± 31*</td>
</tr>
<tr>
<td>KN-62</td>
<td>1026 ± 194</td>
<td>86 ± 15</td>
</tr>
<tr>
<td>Okadaic acid + KN-62</td>
<td>5354 ± 273</td>
<td>449 ± 23*</td>
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<tr>
<td>KN-93</td>
<td>1054 ± 174</td>
<td>91 ± 8</td>
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<tr>
<td>Okadaic acid + KN-93</td>
<td>5394 ± 273</td>
<td>452 ± 19*</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>1051 ± 70</td>
<td>88 ± 6</td>
</tr>
<tr>
<td>Okadaic acid + BAPTA-AM</td>
<td>4836 ± 226</td>
<td>406 ± 19*</td>
</tr>
</tbody>
</table>

*p < 0.05.

Acid, phosphorylation levels are found to be additive when cholera toxin and β-PMA were coapplied or when cholera toxin and okadaic acid were coapplied at maximally effective concentrations (Table II). Although coapplied β-PMA and okadaic acid yield additive reductions in 5HT uptake, cholera toxin does not potentiate the effects of either agent on uptake (data not shown). Interestingly, coapplication of β-PMA, cholera toxin, and okadaic acid is no more effective at augmenting hSERT phosphorylation than the coapplication of any two of these modulators (Table II). Although we did not explore cGMP-linked pathways in detail in the present studies, we also wish to note that the membrane-permeant cGMP analog and activator of PKG, 8-pCPT-cGMP (1 mM, 60 min), also significantly elevated hSERT phosphorylation in 293-hSERT cells with little or no effect on 5HT transport (data not shown). Together these findings reveal the capacity for cyclic nucleotide-activated phosphorylation of hSERT that is uncoupled to regulatory changes in 5HT transport activity in 293-hSERT cells and which appear to be distinct from transporter phosphorylation pathways modulated by PKC and PP1/2A.

DISCUSSION

The transport of 5HT across plasma membranes of platelets, pulmonary endothelium, placenta, and neurons is increasingly described as a tightly regulated process involving both transcriptional and posttranscriptional mechanisms (17). In the nervous system, presynaptic sites of SERT expression are significant distances from nuclear sites of SERT gene transcription, imposing temporal constraints on the ability of cells to modulate transport capacity and thereby affect extracellular 5HT clearance. Even in transfected cells, maturation and surface expression of NE and 5HT transporters takes several hours (37–40); recent in vivo covalent labeling studies on the

Fig. 7. Additivity of β-PMA and okadaic acid-induced hSERT phosphorylation. 293-hSERT cells were labeled with [32P]orthophosphate for 1 h at 37 °C and then incubated with β-PMA (1 μM) and okadaic acid (1 μM), either separately or in combination, for indicated times. The concentrations of β-PMA and okadaic acid used are the maximally effective doses on the phosphorylation of hSERT. RIPA extraction, immunoprecipitation, SDS-PAGE, and autoradiography were performed as described under “Experimental Procedures.” A representative autoradiogram of four separate experiments corresponding to the 32P-labeled hSERT is shown. A, representative autoradiogram of time-dependent phosphorylations of agents added separately or together. B, quantitation of phosphorylation data averaged across three experiments presented as means ± S.E. Asterisks denote values significantly different (p < 0.05, Student’s paired t test) in coactivation experiments from β-PMA treatment alone.

DATs reveal a delay of more than a week to resupply striatal transport sites once they have been irreversibly inactivated (41).

Evidence has accumulated to indicate that receptor and kinase-linked pathways may rapidly up- and down-regulate endogenous SERTs as well as SERTs expressed in heterologous systems (17). We have recently shown (1) that HEK-293 cells stably transfected with hSERT cDNA, like endothelial cells (30), platelets (31), JAR (42, 43), and RBL-2H3 cells (34), and serotonergic neurons (32) support rapid modulation of 5HT transport following activation of PKC. Phorbol esters capable of activating PKC activity rapidly diminish 5HT uptake capacity in 293-hSERT cells, kinetically revealed as a change in 5HT transport Vmax and this down-regulation is blocked by the PKC inhibitor staurosporine (1). hSERT-mediated currents are also down-regulated by β-PMA in a staurosporine-sensitive manner in voltage-clamped 293-hSERT cells, suggesting that regulation is not due to changes in ionic driving forces or the membrane potential (1). Furthermore, reductions in transport capacity are paralleled by a quantitatively similar reduction in cell-surface SERT protein (1). Recently, 5HT uptake capacity changes have been reported with the rat SERT cDNA transiently transfected into COS-7 cells (33), suggesting that this modulation is not unique to the HEK-293 context of our transfection studies. Analogous findings of PKC-dependent modulation of transport capacity have been obtained in studies of native and heterologously expressed dopamine (44–46), NE
FIG. 8. Protein kinase A-dependent and PKC-independent phosphorylation of hSERT. 293-hSERT cells were labeled with [32P]orthophosphate in phosphate-free DMEM for 1 h at 37 °C and then incubated for an additional 120 min with or without activators of adenyl cyclase and/or PKA or PKC inhibitors. Kinase inhibitors were added 30 min prior to the addition of cholera toxin or forskolin. The concentrations used were cholera toxin, 1 μg/ml; forskolin, 1 μM; 8-pCPT-cGMP, 1 mM; KT5720, 1 mM; staurosporine, 200 nM; and bisindolylmaleimide I, 1 μM. RIPA extraction, immunoprecipitation, SDS-PAGE, and autoradiography were performed as described under “Experimental Procedures.” Inset shows a representative autoradiogram of labeling experiments. Averaged data from four separate experiments are presented ± S.E. Asterisks denote values significantly different (p < 0.05, Student’s paired t test) from treatments with no activator or vehicle, performed in parallel.

TABLE II
Coordinate effects of β-PMA, cholera toxin, and okadaic acid-induced phosphorylation of hSERT

| Treatments                      | Band density % control)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>646 ± 45</td>
</tr>
<tr>
<td>β-PMA</td>
<td>2573 ± 190</td>
</tr>
<tr>
<td>Cholera toxin</td>
<td>2556 ± 156</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>3034 ± 192</td>
</tr>
<tr>
<td>β-PMA + cholera toxin</td>
<td>4428 ± 330</td>
</tr>
<tr>
<td>β-PMA + okadaic acid</td>
<td>4417 ± 111</td>
</tr>
<tr>
<td>Cholera toxin + okadaic acid</td>
<td>4341 ± 133</td>
</tr>
<tr>
<td>β-PMA + cholera toxin + okadaic acid</td>
<td>4653 ± 159</td>
</tr>
</tbody>
</table>

* Significantly different from levels observed under basal conditions, Student’s t test, p < 0.05.

| Treatments                      | Band density % control)
<table>
<thead>
<tr>
<th></th>
<th></th>
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</thead>
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<td>β-PMA + cholera toxin</td>
<td>4428 ± 330</td>
</tr>
<tr>
<td>β-PMA + okadaic acid</td>
<td>4417 ± 111</td>
</tr>
<tr>
<td>Cholera toxin + okadaic acid</td>
<td>4341 ± 133</td>
</tr>
<tr>
<td>β-PMA + cholera toxin + okadaic acid</td>
<td>4653 ± 159</td>
</tr>
</tbody>
</table>

a Significantly different from levels observed under basal conditions, Student’s t test, p < 0.05.

Significantly different from the levels observed with the application of single modulators, Student’s t test, p < 0.05.

Not statistically different from the levels observed with the application of single modulators.

(47), γ-aminobutyric acid (48–50), and glycine (51) transporters and thus mechanisms for acute regulation of members of the Na+/Cl−-dependent neurotransmitter transporter gene family may possess many similarities. Members of the gene family share gross topological properties including cytoplasmic NH2 and COOH termini. These latter domains possess a large number of potential targets for protein phosphorylation. Moreover, we have found that the NH2 and COOH termini of SERTs, expressed and purified as GST fusion proteins, are substrates for protein kinases including PKC and PKA (17, 35). These findings increased our interest in exploring the question of whether direct protein phosphorylation of SERTs occurs and how this phosphorylation might effect transporter activity and surface expression.

The present studies demonstrate for the first time the existence of phosphorylated hSERT proteins and support the existence of multiple kinetically and molecularly distinct pathways leading to hSERT phosphorylation. We observe 1) that hSERT protein in 293-hSERT cells is phosphorylated under basal conditions and 2) this phosphorylation is potentiated by addition of the PP1/2A protein phosphatase inhibitors okadaic acid and calyculin A. Although basal phosphorylation is modest with respect to that achieved with phosphatase inhibition, it can be readily perceived when nontransfected cells are immunoprecipitated in parallel. In addition, we document the labeling of hSERT by 3) PKC- and 4) cyclic nucleotide activated kinase (PKA and PKG)-linked pathways. PKC- and PKA-induced hSERT phosphorylation appear additive with each other and with okadaic acid-induced labeling, suggesting that the endogenous phosphorylation protected by phosphatase inhibition is not mediated by either PKC or PKA. Indeed, neither PKC nor PKA inhibitors block the okadaic acid-induced phosphorylation of hSERT at concentrations required to fully abolish PKC or PKA labeling, respectively. Moreover, the kinetics of phosphorylation can be distinguished by activators of these pathways, and distinct effects are observed on transport. Like PKC activation, okadaic acid action leads to a reduction in transport capacity, but a loss of 5HT transport is not seen with PKA activators that phosphorylate hSERT to the same extent. Intracellular calcium and the activity of Ca2+/CaM kinase II have been implicated in acute SERT regulation (42, 52); however, our studies with BAPTA and specific Ca2+/CaM kinase II inhibitors fail to implicate these pathways in okadaic acid-induced phosphorylation. These data lead us to conclude that distinct sites are being phosphorylated as a result of PKC, PKA, and an as yet unidentified endogenous kinase(s).

PKA and PKG activation in 293-hSERT cells leads to rapid phosphorylation of hSERT protein yet no observable functional change in 5HT transport capacity. Although chronically administered cAMP-elevating agents have significant effects on hSERT gene transcription in JAR cells (53, 54), a role for PKA-linked pathways in acute SERT regulation is not evident. PKA-mediated phosphorylation of SERT proteins may reflect artifactual labeling in this heterologous system that never occurs to any significant extent in vivo. However, it is also possible that PKA-mediated labeling is facilitatory to other regulatory signals that we have failed to activate (or cannot) in these cells. In RBL-2H3 cells (34), brain (55, 56), and platelets (57), both nitric oxide and PKG-linked pathways have been implicated in acute, receptor-mediated regulation of 5HT uptake though as yet SERTs have not been shown to be phosphorylated under similar conditions. We find the potent, membrane-permeant analog 8-pCPT-cGMP to elevate SERT phosphorylation although changes in uptake are not evident. As with PKA activation, this labeling may not translate into functional changes due to the heterogeneous or overexpression nature of the current system. Further studies are warranted to determine whether PKA and PKG activation promote the phosphorylation and regulation of SERTs in other cell contexts and whether the sites of phosphorylation overlap with those labeled following PKC activation.

PKC activation leads to both transporter phosphorylation (this study) and transporter redistribution from the cell surface (1). It is not possible, however, to conclude from our findings...
that phosphorylation of transporter protein is causal in the reduction of 5HT uptake since other proteins that may play important roles in transporter trafficking are likely to be targets of PKC-linked pathways as well. However, the close correspondence between dose and time dependence for transport reductions and transporter phosphorylation forces us to consider that at least a signal for membrane redistribution may be direct transporter phosphorylation. Rat and human SERT proteins possess multiple canonical sites for Ser/Thr phosphorylation among which are several PKC sites (18–20, 34). Our own studies with purified cytoplasmic NH₂ and COOH termini (35) reveal both domains to be substrates for PKC (and PKA but not PKG). However, mutation of canonical PKC phosphorylation sites individually did not reduce this phosphorylation but rather enhanced it in several cases (58). Similarly, Sakai and co-workers (33) have described PKC and PKP1/2A-mediated reductions in 5HT transport capacity in transiently transfected COS-7 cells, effects that were not abolished by mutation of canonical PKC sites. Mutation of PKC sites in GAT1 γ-amino butyric acid transporters (49) and GLYT1 glycine transporters (51) also fails to diminish PKC-mediated changes in transport capacity. We suspect that multiple sites may be phosphorylated on cytoplasmic domains by activated PKC (and these sites may interact), such that individual mutations are incapable of diminishing ultimate functional effects. Alternatively, PKC may phosphorylate other kinases and phosphatases that ultimately affect the hSERT phosphorylation state. Currently, we are exploring the merits of these arguments by trying to identify the sites of 32P incorporation and reconstituting the labeling patterns with in vitro extracts to permit kinase identification.

Striking similarities are evident in comparison of our findings with phosphorylation studies of native (45) or heterologously expressed rat DAT proteins (46). DATs expressed in these environments, like hSERTs, exhibit basal phosphorylation that can be augmented by phosphatase inhibition and PKC activation. For both transporters, activity changes are manifested primarily as a reduction in transport Vₘₐₓ. As in our studies, DATs are less sensitive to the PP1/2A/2B inhibitor microcystin than okadaic acid and calyculin A, although this may reflect differential permeability of the compounds in HEK-293 cells. However, both SERT and DAT are insensitive to the calcineurin (PP2B) antagonist cyclosporin A. Presently, we are attempting to define the specific phosphatase complex modulating SERT phosphorylation to differentiate between PP1 and PP2A. An important difference between the DAT and SERT studies is our finding that okadaic acid-induced SERT phosphorylation is staurosporine-insensitive at concentrations required to block β-PMA effects. This might represent a specificity of SERT for a distinct kinase or simply an inability to distinguish PKC and the endogenous kinase in LLC-PK1 cells. Consistent with the latter explanation, Huff and co-workers (45, 46) found additive phosphorylation of DAT proteins with coactivation of β-PMA and okadaic acid. Another striking difference between the DAT and hSERT studies is the evidence we present that PKA and PKG activation, independent of PKC or okadaic acid-linked pathways, leads to SERT phosphorylation. DATs are not phosphorylated by treatment of cells with forskolin; the activation of cGMP-linked pathways has not been reported. Phosphorylation of SERT by PKA- or PKG-linked pathways may reveal modulatory pathways not shared with catecholamine transporters.

Phosphorylation of hSERT induced by activated PKC parallels in time and concentration the loss of 5HT uptake capacity and loss of surface protein. How might the phosphorylation of hSERT protein affect a redistribution of transporter protein from the cell surface? In G-protein-coupled receptors, agonist occupancy and heterologous stimuli lead to the phosphorylation, desensitization, and often, the down-regulation of receptors mediated by internalization (59, 60). Phosphorylated β-adrenergic receptors are recognized by arrestin proteins, targeting them to plasma membrane domains that support clathrin- and dynamin-mediated endocytosis (61). Removal of phosphate from receptors by Ser/Thr phosphatases is thought to assist in the return of fully functional receptors to the cell surface (62). We suspect we have identified pathways that may be analogous to those used for heterologous receptor desensitization. Thus, receptors activating PKC may, in addition to other actions, affect surface distribution of SERT proteins by phosphorylating the transporter and enhancing its internalization rate. Alternatively, PKC activation could slow the rate of transporter insertion in the plasma membrane. Presently, little is known of the dynamics of transporter membrane trafficking. In oocytes, heterologously expressed γ-aminobutyric acid GAT1 transporters appear to be shuttled in and out of the plasma membrane by membrane vesicles dependent on SNARE proteins, a process that is regulated by PKC (50). Interestingly, different pools of PKC appear to be available in oocytes such that exogenous (48) and intracellular (50) PMA treatments yield opposite changes in GAT1 expression. These findings remind us of the potential complexities associated with multiple PKC isoforms (63) and our present lack of knowledge of the cellular compartments where phosphorylation proceeds. It should be very informative to determine whether surface or intracellular SERT proteins are targets of phosphorylation. In 293-hSERT cells, we estimate ~75% SERT proteins are on the cell surface, but without additional studies, we cannot claim that it is the transporter in the plasma membrane that becomes phosphorylated in response to kinase activation or phosphatase inhibition. Interestingly, in preliminary studies, we find that 5HT itself can perturb both basal and PKC-mediated phosphorylation in these cells, suggesting that some or all of the phosphorylation observed may occur on surface resident SERTs accessible to transmitter. In summary, our studies suggest that endogenous transporter phosphorylation may contribute to the rapid regulation of SERT expression and reinforces the idea that phosphorylation may control synaptic signaling by modifying both neurotransmitter release and clearance.

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Phosphorylation and Regulation of Antidepressant-sensitive Serotonin Transporters
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