Evidence for Biphasic Effects of Protein Kinase C on Serotonin Transporter Function, Endocytosis, and Phosphorylation

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Running Title: Dual Actions of PKC on Platelet Serotonin Transporters

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Abbreviations: 5-HT, serotonin; BIM, Bisindolylmaleimide I; CaMKII, calcium calmodulin dependent protein kinase II; DAT, dopamine transporter; hSERT, human serotonin transporter; NET, norepinephrine transporter, NHS-SS-biotin, sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate; PKC, protein kinase C; PP2Ac, protein phosphatase 2A catalytic subunit; SERT, serotonin transporter; β-PMA, phorbol 12-myristate 13-acetate.
ABSTRACT

The serotonin transporter (SERT) regulates serotonin (5-HT) neurotransmission and is a high affinity target for antidepressants and psychostimulants. In the present study, we investigated the mechanisms that contribute to a previously unidentified biphasic regulation of endogenous SERTs expressed in the platelets. Treatment of rat platelets with ß-PMA for 5 min or less resulted in a rapid inhibition of SERT involving changes in intrinsic activity of the transporter (increased Km and decreased Vmax). ß-PMA-treatment for 30 min or more produced a sustained inhibition of SERT with a decrease only in the Vmax. While inhibition of SERT activity was detected from 1 to 45 min following phorbol ester addition, the decrease in surface SERT required at least 30 min of phorbol ester incubation. Increased endocytosis of SERT accounted for the decrease in surface SERT at the later point. PKC-mediated phosphorylation of SERT occurs on the plasma membrane during the initial phase of rapid transporter inhibition, and later on, the phosphorylated SERT enters the intracellular pool. ß-PMA-induced phosphorylation of SERT occurs initially on serine residues(s) and then, on threonine residue(s). The initial serine phosphorylation corresponded to the first phase of rapid inhibition mediated by changes in intrinsic activity and/or silencing of SERT. The later phosphorylation on threonine residue(s) corresponded to the later phase of sustained inhibition mediated by an enhanced endocytosis of SERT. Together, these data reveal that in platelets, SERT function is regulated by PKC in a biphasic manner involving both trafficking-dependent and independent mechanisms, and that these two events occur at distinct phases of transporter phosphorylation.
INTRODUCTION

Serotonin (5-HT) controls many behavioral and physiological functions (Jacobs and Fornal, 1995). 5-HT uptake via the cocaine- and antidepressant-sensitive serotonin transporter (SERT) is essential for serotonergic neurotransmission termination (Barker and Blakely, 1995). Altered SERT expression and two polymorphic regions in SERT promoter have been implicated in multiple forms of psychopathology, including depression, suicide, anxiety, aggression, schizophrenia, alcoholism and drug addiction (Murphy et al., 2004). While clearance of synaptic and extra-synaptic 5-HT appears to be the principal function of SERTs, certain cells, notably platelets, utilize SERTs to acquire 5-HT from the extracellular environment for subsequent release, involving in the processes of platelet activation (Cirillo et al., 1999). Platelets and 5-HT neurons share many common properties, including vesicular monoamine transporters (VMATs), 5-HT release, biochemistry, identical SERT sequences, and 5-HT receptors (Owens and Nemeroff, 1994). In addition, alterations in platelet SERT have been observed in many psychiatric disorders and vascular diseases in which 5-HT has been implicated (Meltzer et al., 1981). Therefore, platelets have been widely used as a peripheral indicator of central 5-HT metabolism and SERT function (Wirz-Justice, 1988).

Previously, using human embryonic kidney cells (HEK-293) stably transfected with human SERT (hSERT), we reported that activation of PKC and/or inhibition of protein phosphatases decreases 5-HT uptake. This down regulation of SERT activity is paralleled by a decrease in SERT surface expression, an increase in SERT phosphorylation and a decrease in SERT-PP2Ac association (Bauman et al., 2000; Ramamoorthy et al., 1998a). We also showed that extracellular SERT substrates attenuate PKC-mediated effects on SERT (Ramamoorthy and Blakely, 1999). Recently, we demonstrated that in HEK-293 cells, PKC activation stimulated
SERT internalization and p38 MAPK inhibition attenuated SERT insertion to the plasma membrane (Samuvel et al., 2005). Interestingly, PKC activation resulted in increased SERT basal phosphorylation and p38 MAPK inhibition resulted in reduced SERT basal phosphorylation. These results suggest that whereas PKC is involved in regulated expression of SERT, p38 MAPK may be involved in maintaining normal/basal expression of SERT. Recently, it has been shown that p38 MAPK activation also induces a trafficking-independent model of SERT stimulation (Zhu et al., 2005). However, many questions still remain unanswered. Is SERT regulation demonstrated in heterologous model systems similar to that of endogenous model systems? Is the PKC-mediated decrease of surface SERT due to endocytic internalization, or decreased exocytic insertion or both? What are the specific PKC isoform(s) involved in SERT regulation? What residues represent SERT phosphorylation sites and are they necessary for regulation? In an attempt to define some of the mechanisms by which PKC acutely regulates native SERT, we characterized the time course and pattern of SERT phosphorylation in parallel with SERT functional regulation using rat platelets as they express endogenous SERTs.

In this study, we demonstrate differential time-dependent molecular events associated with PKC-mediated regulation of endogenous SERTs expressed in the platelets. PKC activation in platelets results in reduced SERT function and enhanced SERT basal phosphorylation. Surprisingly, PKC activation regulates SERT in a biphasic manner, where the initial phase of inhibition occurs independent of trafficking and the later phase is characterized by an enhanced endocytosis. The biphasic inhibition of SERT is accompanied by a sequential phosphorylation of plasma membrane resident SERT initially on serine residue(s) and then on threonine residue(s). Based on the findings, we conclude that the initial phosphorylation on serine residues might be responsible for changes in the intrinsic properties and/or silencing of SERT, and that
the phosphorylation on threonine residues later on might trigger internalization of phosphorylated SERT.
MATERIALS AND METHODS

Materials. Phorbol ester isomers and protease inhibitors were obtained from Sigma Chemical (St. Louis, MO). Okadaic acid was purchased from LC Laboratories/Alexis Biochemicals (San Diego, CA). Bisindolylmaleimide I was purchased from Cal Biochem (San Diego, CA). \[^{3}H\]5-HT (5-hydroxy-[\(^{3}\)H]tryptamine trifluoroacetate), \(^{32}\)PO\(_{4}\) carrier free orthophosphate, Protein A Sepharose beads, ECL reagent and ECL enhanced film were obtained from Amersham Biosciences (Piscatway, NJ). HRP-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Monomeric sterptavidin beads and EZ-link NHS-sulfo-SS-biotin were purchased from Pierce (Rockford, IL). All other reagents were of the highest grade possible from standard commercial sources.

Isolation of platelets: Rat platelets were collected in a tube containing Acid-Citrate-Dextrose from trunk blood following rapid decapitation. All animal procedures were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Blood was centrifuged immediately at 190 x g for 15 min at room temperature to obtain platelet-rich plasma. The platelet-rich plasma was centrifuged at 2500 x g for 15 min at 22˚C, and the resulting platelet pellet was gently resuspended in the assay buffer (140 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES, pH 7.2) and used immediately after counting.

Assay of SERT activity: The activity of SERT in rat platelets was measured by determining the rate of \[^{3}H\]5-HT accumulation. Briefly, \(1 \times 10^{6}\) platelets were incubated in assay buffer containing 100 \(\mu\)M pargyline and 100 \(\mu\)M ascorbic acid with or with out the modulators as
indicated elsewhere. 5-HT uptake assays in triplicate were initiated by the addition of $[^3H]5$-HT (10 nM) for 3 min at 37˚C and terminated by the addition of cold 1 ml assay buffer containing 100 µM imipramine. In some experiments, platelets were washed twice with 10 ml of assay buffer prior to initiation of 5-HT uptake assay. Assay samples were filtered through GF/F glass filters presoaked in 0.03% polyethylenimine. Radioactivity associated with the filters was counted by liquid scintillation spectrometry. Specific uptake was determined by subtracting the amount of $[^3H]5$-HT accumulated in the presence of 0.01 µM fluoxetine.

Metabolic labeling and detection of SERT phosphorylation by immunoprecipitations:
Platelets (1-2 X 10^9/ml) were incubated with 5 mCi/ml of $[^32P]$orthophosphate for 60 min at 37˚C. Stimulation with protein kinase activators was carried out by adding the agents to the same assay mixture and continuing the incubation for appropriate time periods depending on the experiment. At the end of the incubation, the labeled buffer was removed by centrifugation and platelet pellets were washed with cold buffer and solubilized with RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% Na deoxycholate) containing protease and phosphatase inhibitors (1 µM pepstatin A, 250 µM phenylmethylsulfonyl fluoride, 1µg/ml leupeptin, 1 µg/ml aprotinin, 10 mM sodium fluoride, 50 mM sodium pyrophosphate and 1 µM okadaic acid). The solubilized extracts were centrifuged at 20,000 x g for 60 min at 4˚C. Supernatants were precleared by the addition of 100 µl (3 mg) of Protein A Sepharose beads along with pre-immune sera for 1 h at 4˚C, and the clear supernatant was subjected to immunoprecipitation. SERT protein was immunoprecipitated overnight at 4˚C by the addition of SERT specific antibody SR-12 (5 µl of SERT antiserum), or preabsorbed SR-12, preimmune sera and or Protein A Sepharose followed by 1 hr incubation
with Protein A Sepharose beads (3 mg in 100µl in RIPA buffer) at 22˚C. SR-12 is a rabbit polyclonal antiserum raised against the C-terminus amino acid (596-662) sequence of SERT (Ramamoorthy et al., 1998a; Samuvel et al., 2005). The immunoadsorbents were washed and eluted with the addition of 50 µl Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β mercaptoethanol and 0.05% bromophenol blue). Proteins were separated by 6–14% SDS-polyacrylamide gel electrophoresis, and [32P]-labelled SERT protein was detected by autoradiography. Quantitation from digitized autoradiograms was evaluated on multiple film exposures to insure quantitation within the linear range of the film. Band density was quantified using NIH Image 1.60.

**Phosphoamino acids analysis:** Phosphoamino acid analysis was performed as described previously (Ramamoorthy and Balasubramanian, 1989). The region of the gel corresponding to [32P]-labeled SERT was excised and incubated in 0.5 ml of 0.1 M sodium phosphate buffer pH 7.0, containing 1% SDS, 3 mM β-mercaptoethanol and 100 µg histone (as carrier) with continues overnight shaking at 22˚C. The gel was washed once with 0.5 ml of the same buffer without carrier histone. The protein from the combined washings was precipitated with 20% trichloroacetic acid. The precipitated protein was washed once with 10% trichloroacetic acid, twice with acetone and subjected to acid hydrolysis for 90 min in 5.7 N HCl at 110˚C. The HCl was removed by evaporation in vacuo and the samples were subjected to high voltage electrophoresis on cellulose thin-layer plates as described (Ramamoorthy and Balasubramanian, 1989). Standard phosphoamino acids were added to the radioactive samples during hydrolysis and located by ninhydrin spray. The [32P]-phosphoamino acids from SERT were located by autoradiography and aligned with standards.
Quantification of surface SERT by biotinylation. Cell surface biotinylation was performed as described previously (Jayanthi et al., 2004; Ramamoorthy et al., 1998b) with the following modifications: Suspended (1 x 10⁹) platelets were incubated with 0.1 µM β-PMA or vehicle (ethanol) for indicated time periods. Cold assay buffer containing 0.2 mg/ml NHS-SS-biotin was added to the platelet pellet and incubated for 15 min at 4°C. The platelets were washed with cold assay buffer containing 100 mM glycine followed by solubilization with RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% Na deoxycholate) containing protease and phosphatase inhibitors (1 µM pepstatin A, 250 µM phenylmethylsulfonyl fluoride, 1µg/ml leupeptin, 1 µg/ml aprotinin, 10 mM sodium fluoride, 50 mM sodium pyrophosphate and 1 µM okadaic acid). The surface biotinylated proteins were isolated using monomeric avidin beads. SERT levels from total extract, eluted fractions from monomeric avidin beads (surface biotinylated fraction) and non-bound fractions (intracellular fractions) were analyzed by SDS-PAGE (10%), electroblotted to PVDF membrane (Amersham) and probed with SERT antibody as described earlier. Immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham). Subsequently, the blots were stripped and probed with anti-calnexin to validate the surface biotinylation, equal protein load and transfer, and platelet integrity. Values of total, non-biotinylated, and surface SERT protein were normalized using the levels of calnexin in total extract and values were averaged across three or more experiments. Multiple exposures of immunoblots were obtained to insure development within the linear range of the film (Kodak X-AR). The band densities were quantified by integrating band density using computer-assisted densitometry (NIH Image 1.60).
To determine the sub-cellular location of SERT phosphorylation, platelets were metabolically labeled with $[^{32}\text{P}]$-orthophosphate (see above for details) followed by biotinylation as described above except that bound proteins from monomeric avidin beads were eluted using 2 mM free d-biotin. The eluate, unbound fractions, total extractions were subjected to SERT immunoprecipitations and autoradiography.

**Assay of SERT endocytosis by reversible biotinylation.** Reversible biotinylation strategy was used as described recently by us (Jayanthi et al., 2004). Isolated platelets (1 x 10$^9$) were suspended in 1 ml ice-cold assay buffer containing sulfo-NHS-SS-biotin (0.1mg/ml) for 20 min at 4°C. After two washes in assay buffer, two tubes were kept at 4°C with the cold assay buffer with or with out β-PMA (to estimate total surface SERT, efficiency of surface biotin removal) and others were incubated for different times at 37°C in prewarmed assay buffer containing β-PMA (0.1 µM) or vehicle to initiate endocytosis. For each incubation time, samples were kept at 4°C to estimate total surface SERT at a given time period of incubation. Other samples were incubated with 1 ml cold cleaving solution (50 mM MesNa (2-mercaptoethanesulfonic acid), 100 mM NaCl, 50 mM Tris-HCl, pH 8.9) (to estimate internalized SERT) for 20 min and washed three times with assay buffer containing 5mg/ml iodoacetamide. Since MesNa does not penetrate the plasma membrane, the internalized biotinylated SERT is protected from surface-resident SERT and can be selectively detected. The internalized, biotinylated SERT was quantitated based on the loss of sensitivity to MesNa. Biotinylated SERTs that were resistant to reversal of biotinylation was defined as "the amount of SERT endocytosed or internalized". All samples were solubilized using RIPA buffer and biotinylated SERTs were isolated using monomeric avidin beads and then blotted with SERT-specific antibody. To calculate the percent
of SERT internalization, the intensity of the band in the lane where biotinylated platelets stored at 4°C were immediately exposed to MesNa is subtracted as background from all other band densities. In our experimental stripping conditions, the biotinylated SERT present in avidin bound fractions after MesNa treatment was less than 5% of total biotinylated fraction. The level of SERT in MesNa resistant fraction was not altered by keeping cell dishes in ice slurry up to 45 min and between the vehicle and β-PMA treatment. In initial experiments, experiments were optimized for dose, time and pH of the buffer used in stripping procedure with MesNa. We found that MesNa treatment did not completely remove SERT from avidin bound fractions. We always found less than 5% of SERT in avidin bound fractions after MesNa treatment at 4°C. This residual signal may be arising due to the inability of MesNa to access disulfide bonds for reaction as a result of SERT embedding in the plasma membrane.

To determine [32P]-labeled SERT internalization, platelets were metabolically labeled with [32P]-orthophosphate (see above for details) followed by reversible biotinylation as described above except that bound proteins from monomeric avidin beads were eluted using 2 mM d-biotin and subjected to immunoprecipitation of SERT followed by SDS-PAGE and autoradiography.

**Statistical Analyses:** Values are expressed as mean ± SEM. Analysis by one-way analysis of variance was used followed by post hoc testing (Tukey-Kramer, Bonferroni, Dunnett’s test). Student’s t test was performed for paired observations. A value of $p < 0.05$ was considered statistically significant.
RESULTS

PKC Activation Inhibits SERT Activity. Treatment of purified platelets with β-PMA (PKC activator) decreased basal 5-HT uptake. The dose and time-dependence of this inhibition are summarized in Fig. 1 A and B, and shown that β-PMA inhibited SERT activity as rapidly as 1 min and over the concentration from 1 nM to 100 nM. The functional effects of β-PMA were also rapid with 60 to 70% of the maximal inhibition of SERT activity occurring within 5 min. An additional modest decrease in SERT activity was observed after 5 min of incubation with 100 nM β-PMA. Higher concentrations of β-PMA (10 to 500 nM) showed no further decrease in SERT activity and showed a similar time course as observed with 100 nM β-PMA. Therefore, a 100 nM concentration of β-PMA was chosen for subsequent experiments. α-PMA (100 nM), an inactive analogue of β-PMA, did not affect the basal 5-HT uptake (Table 1). Importantly, the inhibitory effect of β-PMA on SERT activity was effectively blocked by the PKC inhibitors, staurosporine (1 µM) and bisindolylmaleimide I (1 µM), but not by the PKA inhibitor, KT5720 (Table 1). In addition, these inhibitors alone had no effect on 5-HT uptake.

Previously, we documented that while SERT substrates, such as 5-HT attenuate PKC-mediated SERT surface down regulation and SERT phosphorylation, SERT antagonist such as SSRIs and cocaine block the effect of 5-HT (Ramamoorthy and Blakely, 1999). Since platelets store and release 5-HT, a possibility of released 5-HT from platelets during β-PMA treatment could dilute radiolabeled 5-HT leading to a rapid loss of radiolabeled 5-HT uptake observed at early treatments of β-PMA (Fig. 1 A). To determine whether released 5-HT has any effect on PKC-mediated rapid inhibition of 5-HT uptake during early stage, platelets were treated with vehicle or β-PMA in the presence and absence of cocaine followed by thorough washings prior
to the initiation of 5-HT uptake. Washing vehicle and β-PMA treated (100 nM, 5 min) platelets prior to 5-HT transport assay exhibited similar effect of β-PMA on 5-HT uptake when compared to unwashed platelets (Fig. 1 C). In addition, incubation of cocaine did not influence 5-HT uptake in the presence and/or absence of β-PMA (Fig. 1C).

Kinetic analysis indicated that β-PMA treatment of platelets affected SERT kinetics differentially based on the time of β-PMA treatment as shown in Table 2. β-PMA treatment for 5 min significantly decreased apparent substrate affinity for 5-HT (Km) and Vmax. However, β-PMA exposure for 30 min decreased Vmax, but had no significant effect on Km (Table 2). Similar results were observed when kinetic analysis was performed on data obtained from using washed platelets (Data not shown).

**PKC Activation Increases SERT Phosphorylation.** To determine whether native SERT proteins expressed in platelets are phosphorylated, platelets were metabolically labeled with $[^{32}\text{P}]$ and immunoprecipitated with SERT-specific antibody (SR-12) and control antisera (Fig. 2 A). SDS-PAGE/autoradiography of immunoprecipitates from β-PMA (0.1 µM, 30 min) exposed platelets revealed a broad band centered at ~100 kDa, the size similar from immunoblots for mature SERT proteins in platelets. The $[^{32}\text{P}]$-labeled 100 kDa was not immunoprecipitated with preabsorbed SR-12, preimmune sera and or Protein A Sepharose (Fig. 2 A). Our studies rely on the specificity of our immunoprecipitations. While there is no SERT knockout rat model available to use as a control or no phospho-specific SERT-antibody available for these studies, we find primarily a single band with direct immunoblotting of platelet extracts. A band of similar size was previously reported in rat midbrain synaptosomes (Samuvel et al., 2005) (data not shown). Moreover, immunoprecipitation studies in transfected cells reveal an absence of
phosphorylation signal with vector transfections and a band of similar size as seen in platelets when cells are transfected with rat SERT cDNA (data not shown). In addition, immunoblot detections show a band of similar size as seen in platelets when cells are transfected with rat SERT cDNA but not in vector transfections. Thus, this SERT-specific antibody (SR-12) has been tested rigorously with respect to its ability to isolate and detect SERT protein.

We tested whether the time course of β-PMA effect shows any correlation between SERT activity and SERT phosphorylation in platelets. Platelets in the assay buffer without any externally added regulators, showed very low levels SERT phosphorylation (Fig. 2 F). Treatment of platelets with 0.1 µM β-PMA increased SERT phosphorylation. The effect of β-PMA was rapid and showed a gradual increase in SERT phosphorylation for up to 10 min followed by a steep increase at 15 min and reached a plateau phase thereafter. (Fig. 2 F & G). The time course of increased SERT phosphorylation paralleled that of SERT inhibition in early time points (1-10 min) of β-PMA treatment. Major (60-70%) inhibition of SERT activity was achieved within this early time point (1-10 min) following β-PMA treatment and thereafter, only a moderate decrease in SERT activity was observed. The initial gradual increase in SERT phosphorylation corresponded to the sharp decrease in SERT activity achieved within 5-10 min and the later steep increase in SERT phosphorylation reaching a plateau corresponded to the later moderate inhibition of 5-HT uptake (Fig. 2 F & G). A similar time course effect of β-PMA on SERT activity and SERT phosphorylation was observed when higher concentrations of β-PMA (1 µM) were used (data not shown). α-PMA (100 nM), an inactive analogue of β-PMA, did not affect SERT phosphorylation (Table 1). The effect of β-PMA was blocked by the PKC inhibitors staurosporine and BIM I, but not by PKA inhibitor, KT5720 (Table 1). Parallel Western blot analysis of SERT and calnexin at each time of β-PMA treatments prior to
immunoprecipitation showed the presence of equal amount of SERT and calnexin proteins in all samples (Fig. 2 B & C). Furthermore, Western blot analysis of SERT from immuno-depleted (after removal of Protein A-Sepharose-SERT immunocomplex) fractions revealed the absence of SERT protein in the immuno-depleted fractions. Western blot analysis of SERT immunocomplexes (immunoprecipitated SERT eluted from Protein A-Sepharose) showed the presence of equal SERT band intensity in the immunoprecipitated fractions at all time points of β-PMA treatments (Fig. 2 D & E). These results suggest that the changes in $[^{32}P]$-labeled SERT observed at different times of β-PMA treatment are not due to uneven use and/or uneven immunoprecipitation of SERT.

**PKC Activation Induces Phosphorylation Initially on Serine Residues Corresponding to Early Phase of SERT Inhibition and Then on Threonine Residues Corresponding to Later Phase of SERT Inhibition.** Since we observed a biphasic reduction in SERT activity following β-PMA treatment, we asked whether β-PMA induces phosphorylation of SERT on different amino acid(s) at different time intervals following treatment. $[^{32}P]$-labeled SERT proteins were immunoisolated from platelets treated with β-PMA either for 5 or 30 min in the presence of phosphatase and protease inhibitors and subjected to gel separation, acid hydrolysis and phosphoamino acid analyses. As shown in Fig. 3, phosphoserine residue(s) but not phosphothreonine or phosphotyrosine residues were detected on phosho-SERT isolated from platelets that were incubated with β-PMA for 5 min. However, both phosphoserine and phosphothreonine residues were detected from platelets that were incubated with β-PMA for 30 min. Densitometry as well as counting of phosphoserine spot showed that there was no increase in the intensity or counts of phosphoserine level between 5 and 30 min of β-PMA treatment (data
not shown). Vehicle treated platelets showed very less SERT phosphorylation and showed no detectable level of phosphoamino acids under 5 or 30 min incubation.

**Biphasic Regulation of SERT Function.** Unlike in 293-hSERT cells, where PKC activation reduces only SERT Vmax without affecting the Km (Qian et al., 1997), in platelets, PKC activation reduced both Vmax and apparent affinity for 5-HT. This could arise as a result of altered catalytic properties or functional silencing of plasma membrane resident SERT and/or altered surface SERT density. The initial increase in SERT phosphorylation (phosphorylation on serine residues) was correlated with a dramatic decrease in SERT activity following the 5 min treatment with β-PMA. There was only a little reduction in SERT activity thereafter, although SERT phosphorylation (phosphorylation on threonine residues) kept increasing during this time (Fig. 2 F & G). These results suggest that the phosphorylation of serine residue(s) influences SERT activity by mechanisms that are different from those mediated by threonine phosphorylation. To examine the mechanisms underlying this biphasic regulation of SERT activity, surface biotinylation experiments were performed to quantitate SERT levels at the cell surface and in the cell interior, both in control and β-PMA treated platelets following 5 and 30 min incubations. SR-12 SERT antibody detects essentially equally both biotinylated and non-biotinylated SERT in platelets (data not shown). Figures 4 A & 4 B show the density of SERT immunoreactivity from biotinylated and non-biotinylated fractions from platelets treated with β-PMA (100 nM) or vehicle. The amount of SERT immunoreactivity from the total extract did not change following 5 min or 30 min treatment with β-PMA. Although, a decrease in SERT activity (Fig. 1 A & Fig. 2 G) was evident following 5 min β-PMA treatment, there was no change either in the cell surface SERT (biotinylated fraction) or in the intracellular SERT (non-
biotinylated) compared to control vehicle treatment. However, a decrease in cell surface SERT (~80% of control) and a simultaneous increase in intracellular SERT immunoreactivity was evident in platelets following 30 min β-PMA treatment (Fig. 4 A & B). Subsequent stripping and reprobing with calnexin, an intracellular marker revealed less than 2% of total calnexin in biotinylated fractions (Fig. 4 A).

**Enhanced SERT Endocytosis Accounts for Decreased Surface SERT Density and Later Phase of SERT Inhibition.** Changes in the rate of endocytosis can alter surface expression and hence functional properties of a protein. To determine whether the β-PMA induced decrease in surface SERT occurring in the later phase was due to increased SERT endocytotic internalization, a reversible biotinylation strategy was exploited. The amount of biotinylated SERT protein was determined for the surface (platelets left at 4°C without cleavage of surface biotin, total biotinylated SERT) and intracellular (after MesNa treatment, internalized biotinylated SERT) pools (Fig. 5). Biotinylation of platelets using NHS-SS-Biotin prior to β-PMA exposure did not affect SERT activity or β-PMA mediated SERT inhibition or SERT immunoreactivity (data not shown). Control experiments performed at 4°C where endocytosis was arrested revealed a background signal of approximately 4-8% compared to platelets incubated at 37°C (Fig. 5 A). This likely represents biotinylated surface proteins that were not completely cleaved by MesNa. This background signal did not appear to be consistently altered by β-PMA treatment. Following 5 min treatment with β-PMA, there was no significant change in the level of biotinylated SERT in MesNa protected fractions compared to vehicle treated control (Fig. 5 A & B). However, a significant increase in the amount of biotinylated SERT in MesNa resistant fractions was observed from platelets treated with β-PMA for 30 min at 37°C.
compared to respective vehicle treated control (Fig. 5 A & B). The percent internalization shown in Fig. 5 B is consistent with a decrease in surface SERT that occurred only at the later phase of SERT inhibition (Fig. 4 A & B).

PKC Mediated SERT Phosphorylation Occurs at the Plasma Membrane. Based on the above results, we predict that the early (5 min) serine phosphorylation of SERT might be occurring at the cell surface, and subsequent threonine phosphorylation may provide a signal for SERT endocytotic internalization and/or occur in parallel with SERT internalization. To test this hypothesis, using metabolically \[^{32}P\]-labeled platelets, we specifically isolated surface biotinylated \[^{32}P\]-labelled SERT from intracellular SERT following \(\beta\)-PMA treatment by sequential SERT isolation using streptavidin-agarose beads and SR-12 SERT antibody. We found \[^{32}P\]-labeled SERT in the plasma membrane (biotinylated) fraction with no detectable \[^{32}P\]-labeled SERT in the intracellular pool (non-biotinylated fraction) following 5 min treatment with \(\beta\)-PMA (Fig. 6 A & B). In contrast, following 30 min of \(\beta\)-PMA exposure, little or no \[^{32}P\]-labeled SERT was detected in the plasma membrane fraction with a parallel increase in the \[^{32}P\]-labeled SERT in the intracellular pool (Fig. 6 A & B). The amount of SERT phosphorylation observed in the total extract at 30 min was approximately equal to the sum of the SERT phosphorylation observed at the cell surface and in the intracellular pool (data not shown). In order to test whether phosphorylated SERT from the plasma membrane indeed is internalized, \[^{32}P\]-labeled SERT was isolated from MesNa resistant fractions as described earlier (Methods) using reversible biotinylation method followed by sequential isolation using streptavidin-agarose beads and SR-12 SERT antibody (Fig. 6 C & D). MesNa treatment in control experiments performed at 4°C where endocytosis was arrested revealed a background
signal of approximately 5-10% of that from platelets incubated at 37°C or from total amount of
\[^{32}\text{P}\]-labeled SERT (Fig. 6 C). This likely represents biotinylated surface proteins that were not
completely cleaved by MesNa. This background signal was unaltered by β-PMA (5 or 30 min)
treatment. Although, a several fold increase in total \[^{32}\text{P}\]-labeled SERT was observed following
5 min β-PMA treatment, no significant increase in \[^{32}\text{P}\]-labeled SERT was detected from MesNa
resistant fractions when compared to the background signal (Fig. 6 C & D). Almost 70% – 80%
of \[^{32}\text{P}\]-labeled SERT was detected in MesNa resistant fraction following treatment with β-PMA
for 30 min at 37°C compared to total \[^{32}\text{P}\]-labeled SERT (Fig. 6 C & D).
DISCUSSION

The elucidation of the mechanisms underlying SERT regulation and signal transduction pathways are crucial to the understanding of SERT function in 5-HT homeostasis. PKC is a well-established regulator for SERT activity (Anderson and Horne, 1992; Qian et al., 1997; Ramamoorthy et al., 1998a). Early studies using heterologously expressed hSERT in 293-HEK cells showed that β-PMA increased SERT phosphorylation with parallel decreases in the Vmax for uptake of 5-HT and surface SERT density (Qian et al., 1997; Ramamoorthy et al., 1998a). Triggering SERT activity by providing SERT substrates and co-ions attenuated this PKC-mediated decrease in SERT phosphorylation and SERT surface density (Ramamoorthy and Blakely, 1999). 5-HT suppresses SERT phosphorylation, suggesting the surface pool is phosphorylated but this has not been confirmed directly. The results of the present study demonstrate that, similar to heterologously expressed SERT, native SERT expressed in platelets is inhibited by PKC activation and this inhibition is accompanied by a decrease in surface expression of SERT protein. However, analysis of 5-HT transport kinetics indicated a significant decrease in both the transport capacity (Vmax) and the apparent affinity for 5-HT after 5 min β-PMA treatment. Interestingly, 30 min of β-PMA treatment caused a reduction only in Vmax. The change in the apparent substrate affinity suggests that PKC activation results in altered intrinsic transport properties. Future studies examining endocytotic-signal and/or PKC-phosphorylation defective SERTs should reveal the difference between intrinsic and trafficking mediated SERT regulation.

Surface biotinylation studies indicated no change in cell surface SERT level after 5 min exposure to β-PMA, but a significant decrease in cell surface SERT level following a 30 min
incubation. These results further support the idea that early inhibition of SERT is due to altered intrinsic transport properties and/or silencing of 5-HT transport rather than trafficking changes. The later inhibition was due to a redistribution of SERT protein from the plasma membrane to cytosolic fraction via increased SERT endocytosis. Although we observed a rapid ~90% inhibition of 5-HT uptake following β-PMA treatment, kinetic analysis showed a 50-60% reductions in Vmax values. It is probable that β-PMA treatment could cause release of 5-HT from platelets and influence the effect of β-PMA on SERT regulation. Earlier studies have shown that SERT substrates such as 5-HT attenuate PKC effects on SERT regulation and the effect of 5-HT is blocked by SERT antagonists such as cocaine (Ramamoorthy and Blakely, 1999; Whitworth et al., 2002). Thus, the observed rapid decrease in 5-HT uptake, changes in 5-HT uptake kinetics, SERT surface expression and SERT phosphorylation within the early β-PMA treatment could represent a composite of influence of released 5-HT on PKC-mediated SERT effect from platelets. However, in our current study with washed platelets, and or β-PMA treatment in the presence of SERT blocker cocaine does not significantly affect the β-PMA effect on 5-HT uptake (Fig. 1C). If the rapid early inhibition of 5-HT by β-PMA were due to enhancement of release of 5-HT from platelets by β-PMA influencing PKC-mediated regulation of 5-HT uptake, there would have been a lesser inhibition in washed platelets and that cocaine should prevent this effect. Though the possibility that the influence of released 5-HT from platelets on SERT as well as 5-HT receptors could not be completely ruled out in the current study, the data support the assertion that the early rapid inhibition of 5-HT uptake due to β-PMA treatment may not be associated with release of 5-HT from platelets.

Interestingly, the early inhibition was accompanied by an increase in SERT phosphorylation on serine residues with the later inhibition being associated with an increase in
SERT phosphorylation on threonine residues. Phosphorylation of serine and threonine residues has been described for DAT expressed in rat brain synaptosomes and cells transfected with DAT following PKC activation (Foster et al., 2002; Granas et al., 2003; Lin et al., 2003). While it is not known whether direct SERT phosphorylation modulates SERT activity, trafficking, or both, the current data suggest that the dual stage SERT phosphorylation may have functional importance in SERT regulation. Changes in transport kinetics (both transport capacity and substrate apparent affinity) and the dual stage phosphorylation of SERT suggests that the early phosphorylation on serine residues might be involved in changing the intrinsic transport properties having a major role in SERT inhibition. The later phosphorylation on threonine residues might be involved in SERT internalization with less direct effect on transport function. Examination of the time course of internalization of phosphorylated SERT indicated that up to 5 min, phosphorylated SERTs are present only at the plasma membrane level, and internalized phospho-SERT could be isolated after 5 min. It is therefore possible that early SERT phosphorylation on serine residues may not only perturb SERT function (render SERT nonfunctional) but may also bring about conformational changes exposing threonine residues on SERT proteins for later phosphorylation by PKC or another kinase downstream of PKC. Thus, phosphorylation on threonine residues may provide a signal for SERT internalization.

Activation of PKC generally leads to inhibition of transporter-mediated uptake and sequestration of transporter protein from the cell surface to intracellular compartment (Beckman and Quick, 1998; Ramamoorthy, 2002; Robinson, 2003; Zahniser and Doolen, 2001). However, recently, Lin et al showed a change in DAT cell surface expression following PI3K and MEK1/2 kinase modulation but not by PKC activation even though there was a ~30% inhibition of DA uptake. This suggests that changes in transporter activity cannot always be accounted for by
changes in surface transporter level (Lin et al., 2003). PKC activation also results in transporter phosphorylation suggesting an association between transporter phosphorylation and internalization (Foster et al., 2003; Lin et al., 2003; Ramamoorthy et al., 1998a; Vaughan, 2004). Recently it has been showed that DAT exhibits normal PKC mediated transporter kinetics and trafficking patterns even when PKC mediated DAT phosphorylation is eliminated by mutation of predicted intracellular PKC sites on DAT (Granas et al., 2003) suggesting that PKC-mediated DAT phosphorylation and down-regulation are two independent phenomena. It is possible that PKC-mediated SERT phosphorylation and down regulation may be independent events occurring in parallel. While it is not known whether direct SERT phosphorylation is essential for transporter down regulation, it is possible that transporter phosphorylation may have distinct roles in different transporters. Indeed, the N- and C- termini that contain the majority of consensus phosphorylation sites are poorly conserved among the members of monoamine transporter family (Vaughan, 2004). SERT and DAT also differ in the effect of substrate and inhibitors on their PKC-mediated down-regulation (Chi and Reith, 2003; Daniels and Amara, 1999; Ramamoorthy and Blakely, 1999; Saunders et al., 2000). Experiments with molecular mutant(s) of SERT phosphate acceptor sites are currently underway to establish any direct link between PKC-linked SERT phosphorylation and inhibition of 5-HT transport.

One quantitative aspect of the data in the present study is noteworthy. More than two-thirds (majority) of the inhibition of SERT activity was achieved within 5 min of β-PMA treatment (Fig. 1), a time when no detectable decreases in surface SERT protein were evident (Fig. 4). After 30 min, when PKC activation decreased SERT surface expression (Fig. 4), a modest additional decrease in SERT activity occurred (Fig. 1 & 2). Although the activity and surface density data were not obtained in the same platelets, it is evident that the decrease in
SERT activity at 5 min is due to decreased intrinsic SERT transport activity. There is convincing evidence that transporter proteins exist in association with partner proteins such as PP2A, PICK1, Hic-5, syntaxin 1A (Bauman et al., 2000; Carneiro et al., 2002; Quick, 2003; Sung et al., 2003; Torres et al., 2001). Currently there is no evidence that binding of these partner (transporter associated) proteins to transporter per se alters transporter phosphorylation. However, it is possible that association of these proteins with the transporter can be a mechanism of altering transporter activity without changing transporter abundance in the plasma membrane. As indicated above, this may be mediated by SERT phosphorylation on serine residues, binding to associated proteins, or both. It has recently been demonstrated that activation of p38 MAPK-linked pathways in multiple models, including platelets, increases 5-HT transport activity without enhancing SERT trafficking, an effect linked to an increase in 5-HT binding affinity (Zhu et al., 2005). Our studies with PKC activation may represent antagonism of this same process or may involve population of a novel inactive state. Our studies also indicate the existence of a correlation between SERT activity, SERT phosphorylation and SERT internalization. However, the stoichiometry of SERT phosphorylation and the mechanisms underlying this relationship remain to be elucidated.

In summary, the data from the present study suggest a model of biphasic regulation of SERT activity by PKC activation involving dual mechanisms. Immediate inhibition is mediated by changes in intrinsic activity of SERT and/or silencing SERT function and is associated with serine phosphorylation. A more sustained inhibition is produced by enhanced endocytic removal of SERT from the plasma membrane. Presently the fates of the remaining surface transporters and the ones that have undergone endocytosis are unknown. We speculate two possible ways of biphasic SERT regulation that are consistent with the empirical observations. One possibility is
that serine residues on certain fractions of SERT undergo phosphorylation resulting in dramatic reductions in transport via yet unidentified mechanisms at 5 min. At 30 min, the serine phosphorylated SERT fraction undergoes further phosphorylation on threonine residues triggering SERT endocytosis. Internalized SERT may be dephosphorylated by an associated phosphatase triggering exocytotic insertion into the plasma membrane. The rate of exocytotic insertion of SERT (now dephosphorylated and reactivated) might balance the rate of endocytosis of SERT resulting in very little additional decrease in SERT function. The second possibility is that serine residues on all of SERT undergo phosphorylation resulting in dramatic reduction in transport. A restricted population of serine phosphorylated SERT then undergoes phosphorylation on threonine residues triggering internalization. The remainder of serine phosphorylated SERT might recover its function via reversal of phosphorylation, binding to associated proteins, or both. In both ways, total cellular SERT phosphorylation remains elevated at 30 min due mainly to the intracellular endocytosed pool of SERT.

Platelets express 5-HT1A receptors, and thus precludes an examination of the influence of SERT-mediated 5-HT effect on PKC mediated SERT sequential phosphorylation and trafficking. Further studies are needed to understand the time dependent- differential influence of β-PMA on this complex SERT regulation in the presence of SERT substrates via SERT and 5-HT receptors. The active transport of 5-HT by platelet SERT is thought to be important in maintaining the circulating concentration of 5-HT below the levels required to activate vascular smooth cells and platelet 5-HT receptors (Musselman et al., 2002; Nemeroff et al., 1998; Stoltz, 1985). Therefore, the above findings suggest that SERT function in platelets may be regulated by kinases and phosphatases linked to endogenous receptors and that this regulation may play a role in maintaining blood 5-HT levels. Any perturbations in this homeostatic regulation may
contribute to elevated platelet 5-HT levels reported in some autistic subjects (Anderson et al., 1990; Cook et al., 1997; Cook and Leventhal, 1996).
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REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Effect of β-PMA on 5-HT uptake in isolated rat platelets.  

A, rat platelets were preincubated with vehicle, 0.5 µM β-PMA for the times indicated  
B, treated with indicated concentrations of β-PMA for 45 min and  
C, treated with indicated agents followed by washing,  
before 5-HT uptake assays.  5-HT (10 nM, 3 min) uptake assays were performed as described  
under the “Materials and Methods” section.  Data are the averaged from three separate  
experiments and normalized to vehicle (at each time point, open bar) and the mean values ±  
S.E.M are given.  Nonspecific uptake was defined as the uptake in the presence of 0.01 µM  
fluoxetine and subtracted from the total accumulation to yield specific uptake.  * (p <0.05) and  
** (p <0.01) denote values significantly different from vehicle controls, ANOVA with Tukey-  
Kramer post-hoc analysis.

Fig. 2. Effect of β-PMA on SERT phosphorylation and 5-HT uptake.  

A, rat platelets (1-2 x 10^9/ml) were metabolically labeled with [\(^{32}\)P]orthophosphate for 60 min at 37˚C and treated with  
0.1 µM β-PMA for 30 min.  RIPA extraction, immunoprecipitation, SDS-PAGE and  
autoradiography were performed as described under the “Materials and Methods” section.  The  
[\(^{32}\)P]-labelled SERT (~100 kDa) is specifically immunoprecipitated with SERT-immune sera  
SR-12.  Parallel experiments were performed using pre-immune sera, preabsorbed SR-12 and  
protein A beads as to validate specificity of SERT specific antibody SR-12.  Note that no [\(^{32}\)P]-  
labelled SERT band is observed when pre-immune serum or Protein A Sepharose or preabsorbed  
SR-12 antibody was used.  B-E, rat platelets (1-2 x 10^9/ml) were metabolically labeled with  
[\(^{32}\)P]orthophosphate for 60 min at 37˚C and treated with 0.1 µM β-PMA and vehicle for
indicated times. RIPA extraction, Western blot analysis, immunoprecipitation, SDS-PAGE and autoradiography were performed as described under the “Materials and Methods” section. B, SERT and C, calnexin Western blots from total extract before subjected to SERT immunoprecipitation (ipp). D, SERT Western blot from samples after SERT immunoprecipitation, E, SERT Western blot from SERT immunoprecipitation complex F, an autoradiogram of $^{32}$P-labeled SERT and G, the bar graph shows the relative intensity of $^{32}$P-labeled SERT and 5-HT uptake as in Fig. 1 A. Each blot is a representative of four separate experiments. Data are presented as mean values ± S.E.M. *, # ($p < 0.05$) and **, ## ($p < 0.01$) indicate significant differences compared to 0 min treated controls, ANOVA with Bonferroni post-hoc analysis.

**Fig. 3. Effect of β-PMA on phosphoamino acid composition of SERT.** $^{32}$P-labeled platelets as in Fig. 2 were treated with 0.1 µM β-PMA and vehicle for 5 and 30 min. The region corresponding to $^{32}$P-labeled SERT bands were excised from gel, eluted, acid hydrolyzed and subjected to high voltage electrophoresis. Details are given in the “Materials and Methods” section. A representative autoradiogram showing the presence of $^{32}$P-phosphoserine and $^{32}$P-phosphothreonine is presented. Locations of ninhydrin-stained standard phosphoamino acids (PS-phosphoserine; PT, phosphothreonine; PY, phosphotyrosine) are indicated. Due to low levels of $^{32}$P-labeled SERT in vehicle treated samples, there were no detectable $^{32}$P-labelled phosphoamino acids.

**Fig. 4. Effect of β-PMA on surface expression of SERT.** Rat platelets were treated with 0.1µM β-PMA and vehicle for 5 and 30 min. Surface proteins were biotinylated and recovered
from solubilized extracts by monomeric avidin-beads and SERT was detected by immunoblot as described under “Materials and Methods” section. A, a representative SERT immunoblot of four separate experiments is shown. B, quantitative analysis of SERT band densities. SERT proteins were quantified using NIH image, and the densities of SERT band from three separate experiments are presented as mean ± S.E.M. * (p < 0.05) indicates significant changes in cell surface and intracellular SERT following β-PMA compared to vehicle treatment for 30 min. # (p < 0.05) denotes significant changes compared to β-PMA and vehicle treatment for 5 min. (ANOVA followed by Bonferroni method).

Fig. 5. Effect of β-PMA on SERT endocytosis. A, platelets were surface-biotinylated using cleavable biotin (sulfo-NHS-SS-biotin), incubated for 5 and 30 min at 37°C in the presence or absence 0.1 µM β-PMA for endocytosis, and the remaining surface-accessible biotin was cleaved and removed with MesNa treatment (see “Materials and Methods” section for details). The internalized biotin-bearing proteins were recovered by avidin agarose isolation, and SERT protein was quantified by immunoblot. Some surface-biotinylated platelets were kept at 4°C throughout the procedure and biotinylated SERT were analyzed before (signal represents total SERT biotinylated on the surface) and after treatment (signal represents the effectiveness of MesNa cleaving surface biotin-SS-linked proteins) with MesNa. B, quantitation of Biotinylated SERT bands. Biotinylated SERT was quantified, and the percentages internalized (compared to total surface SERT) in three separate experiments are shown as the mean ± S.E.M. Values from β-PMA treatments were compared with vehicle treatments using Student’s t-test: (*, p < 0.05).
Fig. 6. Subcellular location of phosphorylation of SERT: A, SERT phosphorylation on the surface: Rat platelets were labeled with $[^{32}\text{P}]-$orthophosphate and treated with 0.1 µM β-PMA or vehicle for 5 and 30 min followed by surface biotinylation as in Fig. 4. Solubilized proteins were subjected to two successive isolations. Biotin-linked proteins were separated from non-biotin-linked proteins by monomeric avidin beads and bound proteins were eluted using 2 mM d-biotin. $[^{32}\text{P}]-$labeled SERT proteins from eluate, unbound fractions, total extracts were immunoprecipitated using SERT specific SR-12 antibody and subjected to SDS-PAGE and autoradiography. B, quantitation of $[^{32}\text{P}]-$labeled SERT. Data are presented as mean values ± S.E.M. * ($p<0.05$) and ** ($p<0.01$) indicate significant differences compared to vehicle controls (ANOVA followed by Bonferroni method). C, internalization of $[^{32}\text{P}]-$labeled SERT: $[^{32}\text{P}]-$orthophosphate labeled platelets were surface biotinylated using cleavable biotinylating agent Sulfo-NHS-SS-biotin at 4°C. Platelets were then treated with 0.1 µM β-PMA or vehicle for 5 and 30 min. followed by biotin stripping as in Fig. 5. Two successive isolations of $[^{32}\text{P}]-$labeled SERT from the avidin bead eluates and SERT immunoprecipitations and autoradiography are as described in Fig. 6A. D, quantitation of internalized $[^{32}\text{P}]-$labeled SERT: *** ($p<0.001$) indicates significant change in the level of MesNa resistant $[^{32}\text{P}]-$labeled SERT compared to 5 min β-PMA treatment. Experiment was repeated three times with essentially equivalent results.
Table 1. Phosphorylation and Regulation of SERT in Platelets Through PKC-Linked Pathways: SERT activity and SERT phosphorylation were performed as described under “Experimental Procedures”. Platelets were exposed to β-PMA for 5 min and 30 min at 37˚C. Kinase inhibitors were added to the incubation mixture 15 min prior to the addition of β-PMA. The concentrations used were β-PMA, 100 nM; α-PMA, 100 nM; staurosporine, 500 nM; Bisindolylmaleimide I, 250 nM; KT5720, 1µM. Controls (vehicle) received same concentrations of vehicles. Experiments for SERT activity were conducted in triplicates and the mean values ± SEM from three different experiments were given. For SERT phosphorylation, autoradiograms from three different experiments were scanned and the mean values ± SEM were given as arbitrary units. Data are expressed as percentage of vehicle. Data were analyzed using one-way factorial ANOVA with post hoc Tukey-Kramer tests. Stau, staurosporine.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>β-PMA preincubation time</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
<td>30 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SERT- activity (% of control)</td>
<td>SERT-phosphorylation (band density, % of control) (arbitrary units)</td>
<td>SERT- activity (% of control)</td>
</tr>
<tr>
<td>vehicles</td>
<td>100.00 ± 5.23</td>
<td>100.00 ± 10.18</td>
<td>100.00 ± 8.92</td>
</tr>
<tr>
<td>β-PMA</td>
<td>32.24 ± 3.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>326.64 ± 33.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.76 ± 3.34&lt;sup&gt;a&lt;/sup&gt;*</td>
</tr>
<tr>
<td>α-PMA</td>
<td>99.26 ± 4.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>105.26 ± 5.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100.00 ± 1.53&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stau</td>
<td>105.26 ± 3.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>98.84 ± 12.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>109.35 ± 2.90&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-PMA+stau</td>
<td>98.87 ± 3.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>115.94 ± 17.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100.09 ± 3.12&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>BIM I</td>
<td>107.57 ± 0.86&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100.58 ± 9.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>104.57 ± 1.11&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>β-PMA+BIM I</td>
<td>100.13 ± 2.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>114.70 ± 12.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>97.24 ± 7.51&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>KT5720</td>
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<td>101.24 ± 3.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100.02 ± 4.56&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-PMA+KT5720</td>
<td>30.35 ± 3.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>299.98 ± 11.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.03 ± 0.84&lt;sup&gt;a&lt;/sup&gt;*</td>
</tr>
</tbody>
</table>

<sup>a</sup> p <0.0001 significantly different from vehicles  
<sup>b</sup> p <0.001 significantly different from β-PMA  
<sup>c</sup> Not significantly different from vehicles  
<sup>#</sup> p <0.001 significantly different from β-PMA induced SERT phosphorylation at 5 min preincubation time  
<sup>*</sup> Not significantly different from β-PMA induced SERT activity inhibition at 5 min preincubation time
Table 2. Time course effect of β-PMA on $V_{\max}$ and $K_m$ values of 5-HT uptake. Platelets were exposed to β-PMA (0.1 µM) for 5 min and 30 min at 37°C. Controls (vehicle) received same concentrations of vehicles. Experiments for SERT activity were conducted in triplicates as described under “experimental procedures” and the mean values ± SEM from three different experiments were given. Nonlinear curve fits of data for uptake used the generalized Michaelis-Menten equation to obtain $V_{\max}$ and $K_m$ values for 5-HT uptake. Data were analyzed using one-way ANOVA with post hoc Tukey-Kramer tests.

<table>
<thead>
<tr>
<th>β-PMA Treatment</th>
<th>$K_m$ (nM)</th>
<th>$V_{\max}$ (pmol/10⁶ platelets/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>β-PMA</td>
</tr>
<tr>
<td>5 min</td>
<td>54.33 ± 2.18</td>
<td>164.22 ± 1.96*</td>
</tr>
<tr>
<td>30 min</td>
<td>56.12 ± 1.25</td>
<td>64.76 ± 1.27#</td>
</tr>
</tbody>
</table>

* $P<0.05$ versus vehicle.
# $P<0.05$ versus β-PMA, 5 min
Figure 2

A) 32P-labeled SERT
B) SERT (before ip)
C) Calnexin (before ip)
D) SERT (after ip)
E) SERT (in ip)
F) 32P-labeled SERT

β-PMA preincubation time (min)

G) 5-HT uptake
- 5-HT uptake (% of control)
- Phospho-SERT band density (% increase over 0 min)

Specific 5-HT uptake (% of control)

β-PMA preincubation time (min)
Figure 3

The figure shows a gel image with three treatments: vehicle, 5min, 30min, and β-PMA, 5min, 30min. The gel is labeled with PS, PT, and PY.
Figure 4

A

<table>
<thead>
<tr>
<th></th>
<th>SERT 5 min</th>
<th>SERT 30 min</th>
<th>Calnexin 5 min</th>
<th>Calnexin 30 min</th>
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<tbody>
<tr>
<td></td>
<td>vehicle</td>
<td>β-PMA</td>
<td>vehicle</td>
<td>β-PMA</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>Biotinylated (surface)</td>
<td>Non-biotinylated (intracellular)</td>
<td></td>
</tr>
</tbody>
</table>

B

SERT band density (% of vehicle)

- vehicle, 5 min
- β-PMA, 5 min
- vehicle, 30 min
- β-PMA, 30 min

Surface (Biotinylated) Intracellular (Non-Biotinylated)
**Figure 5**

**A**

- **Biotinylated-surface SERT** (at 4°C before MesNa)
- **Biotinylated-SERT** (at 4°C after MesNa)
- **Biotinylated-internalized SERT** (at 37°C after MesNa)

<table>
<thead>
<tr>
<th></th>
<th>5 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>β-PMA</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
</tbody>
</table>

**B**

- **% SERT internalized**

- **vehicle**
- **β-PMA**

- 5 min: ![Image](image5) ![Image](image6)
- 30 min: ![Image](image7) ![Image](image8)

*Note: The * symbol indicates statistical significance.*