Phosphorylation of the Norepinephrine Transporter at Threonine 258 and Serine 259 Is Linked to Protein Kinase C-mediated Transporter Internalization*

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Recently, we have demonstrated the phosphorylation- and lipid raft-mediated internalization of the native norepinephrine transporter (NET) following protein kinase C (PKC) activation (Jayanthi, L. D., Samuvel, D. J., and Ramamoorthy, S. (2004) J. Biol. Chem. 279, 19315–19326). Here we tested an hypothesis that PKC-mediated phosphorylation of NET is required for transporter internalization. Phosphoamino acid analysis of 32P-labeled native NETs from rat placental trophoblasts and heterologously expressed wild type human NET (WT-hNET) from human placental trophoblast cells revealed that the phorbol ester (β-PMA)-induced phosphorylation of NET occurs on serine and threonine residues. β-PMA treatment inhibited NE transport, reduced plasma membrane hNET levels, and stimulated hNET phosphorylation in human placental trophoblast cells expressing the WT-hNET. Substance P-mediated activation of the Gαq-coupled human neurokinin 1 (hNK-1) receptor coexpressed with the WT-hNET produced effects similar to β-PMA via PKC stimulation. In striking contrast, an hNET double mutant harboring T258A and S259A failed to show NE uptake inhibition and plasma membrane redistribution by β-PMA or SP. Most interestingly, the plasma membrane insertion of the WT-hNET and hNET double mutant were not affected by β-PMA. Although the WT-hNET showed increased endocytosis and redistribution from caveolin-rich plasma membrane domains following β-PMA treatment, the hNET double mutant was completely resistant to these PKC-mediated effects. In addition, the PKC-induced phosphorylation of hNET double mutant was significantly reduced. In the absence of T258A and S259A mutations, alanine substitution of all other potential phosphosites within the hNET did not block PKC-induced phosphorylation and down-regulation. These results suggest that Thr-258 and Ser-259 serve as a PKC-specific phospho-acceptor site and that phosphorylation of this motif is linked to PKC-induced NET internalization.

The norepinephrine (NE)2 transporter (NET) regulates noradrenergic signaling by mediating the clearance of NE and is an important target for antidepressants and psychostimulants (2–5). NE signaling is linked to behavioral arousal (6) and is affected in stress-related paradigms linked to depression (7, 8). NE acutely inhibits nociceptive transmission, including that mediated by SP (NK1), potentiates opioid analgesia, and underlies part of antinociceptive effects of tricyclic antidepressants (9). Various biologic stimuli regulate NE signaling, and alterations in NE signaling, including NE clearance and NET density, are observed in cardiovascular diseases and brain disorders (10–13). Recent studies provided evidence for protein kinase C (PKC)-mediated regulation of NET function attributed to alterations in NET surface redistribution (1, 14, 15). Signals mediated through G-protein-coupled receptors are a likely trigger for PKC-mediated regulation of NET, and such receptors are abundant on neuronal and non-neuronal cells.

Human placenta expresses both SERT and NET (16–18), and we have developed trophoblast cultures from the rat placenta that robustly express endogenous NET (19). Placenta also expresses several receptors, including receptors for peptides such as insulin, SP, and NKB, neurotransmitters, and growth factors (20–25). Our recent study using rat placental trophoblasts demonstrated that PKC activation stimulates lipid raft-mediated internalization of native NET (1). The presence of NET in lipid rafts suggests that signaling machinery specific to lipid rafts may be linked to PKC-mediated NET down-regulation. The PKC-mediated internalization of NET occurs in parallel to an enhanced phosphorylation of the transporter. However, whether PKC directly phosphorylates NET, and whether NET phosphorylation is required for NET transport regulation remained uninvestigated. Studies using phospho-site mutants of transporters have provided positive as well as negative correlations between phosphorylation and transporter functional regulation (26–29). NET protein contains multiple consensus

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2 The abbreviations used are: NE, norepinephrine; NET, norepinephrine transporter; DAT, dopamine transporter; GAT1, GABA transporter; SERT, serotonin transporter; SP, substance P; HTR, human placental trophoblast; β-PMA, β-phorbol 12-myristate 13-acetate; PKC, protein kinase C; WT-hNET, wild type human NET; ANOVA, analysis of variance; MesNA, sodium 2-mercaptoethanesulfonate Mes, 4-morpholinooethanesulfonic acid; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; sulfo-NHS-Ss-biotin, biotin sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate; DM, T258A/S259A double mutation.
sites for several kinases, including PKC, that are distinct from those present in DAT or SERT, and therefore, NET may be regulated by mechanisms that are different for those of DAT and SERT. Importantly, studies that demonstrate altered response to second messenger and/or kinase-mediated regulations in human variants of monoamine transporters (30–32) justify the search for underlying mechanisms of transporter phosphorylation regulating amine transport in normal physiology and pathophysiology.

In this study, we tested two hypotheses as follows: 1) activation of the G_{

Alabaster, AL). All other chemicals were from Sigma unless otherwise indicated.

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**EXPERIMENTAL PROCEDURES**

**Materials**—Human placental trophoblast (HTR) cell line was a kind gift from Dr. Charles H. Graham, Queen’s University, Ontario, Canada. Polyclonal NET antibody was the same used in our earlier study (1). Mouse monoclonal antibody to hNET was from Monoclonal Antibody Technologies (Atlanta, GA). His\_\_ monocalonal antibody was from BD Biosciences. PKC\_\_ was from Monoclonal Antibody Technologies (Albaster, AL). All other chemicals were from Sigma unless otherwise indicated.

**Site-directed Mutagenesis**—The cDNA encoding the wild type His-tagged human NET (WT-hNET) in pCDNA3 was kindly provided by Dr. Randy Blakely (Vanderbilt University School of Medicine, Nashville, TN). The hNET cDNA was subcloned into the mammalian vector pIRE\_S containing blasticidin resistance gene. Phosphosite mutants of hNET, which include single-site mutants harboring T258A or S259A, the double mutant harboring T258A and S259A in intracellular loop 2 (ICL2), the multisite mutant (N’ + C’ + S502A) harboring T19A, T30A, T58A (in the N terminus), S579A, T580A, S583A (in the C terminus) along with S502A (in ICL5), and the other multisite mutants (N’ + C’ + S502A/T258A/S259A) harboring T19A, T30A, T58A, S579A, T580A, S583A, S502A, along with T258A and S259A, were generated by PCR based mutagenesis in this background using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutations were confirmed by restriction enzyme mapping and automated sequencing of the entire DNA sequences on both strands.

**Cell Cultures and Transfections**—Isolation and culture of rat placental trophoblasts were performed essentially as described earlier (1). HTR cells were cultured in a mixture of RPMI 1640 (Mediatech-Cellgro, VA) supplemented with 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (100 \mu g/ml). Cells seeded in 24-well cell culture plates (100,000 cells/well) or 12-well plates (200,000 cells/dish or well) were allowed to grow in an atmosphere of 95% air, 5% CO\(_2\) and used for the experiments. In single DNA transfections, HTR cells were transiently transfected with WT-hNET cDNA or mutant hNET cDNAs (0.5 \mu g of WT-hNET or hNET mutants/well in 24-well plates and 1 \mu g of hNET or hNET mutants/well in 12-well plates) at a 1:1 DNA ratio using FuGENE 6 transfection reagent (Roche Diagnostics). In cotransfections, HTR cells were transfected with WT-hNET cDNA plus hNK-1 receptor cDNA in pCI\_N vector (28) or mutant hNET cDNAs plus hNK-1 receptor cDNA (0.25 \mu g of WT-hNET or hNET mutants plus 0.25 \mu g of hNK-1 receptor/well in 24-well plates and 0.5 \mu g of hNET or hNET mutants plus 0.5 \mu g of hNK-1 receptor/well in 12-well plates). The amount of hNET double mutant cDNA was doubled in some transfections to equalize mutant transporter expression level with that of WT-hNET as indicated elsewhere. However, it should be noted that the expression levels were similar but not identical. Cell cultures were maintained for 24 h prior to transfections and grown for 48 h prior to experiments.

**Phosphoamino Acid Analysis**—Rat placental trophoblasts and HTR cells transfected with the WT-hNET were metabolically labeled with \(^{32}\)P as described before (1). Metabolically labeled cells were treated with 0.5 \mu M \beta-PMA and \(^{32}\)P-labeled NETs from rat placental trophoblasts or \(^{32}\)P-labeled hNET from HTR cells were isolated by immunoprecipitations with NET-specific antibody as described previously (1). Immunoprecipitated \(^{32}\)P-labeled NETs were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes (acid base-resistant; Immobilon). Following autoradiography of blots, phosphorylated NET bands were excised out and pooled from three separate experiments before hydrolyzing with 5.7 M HCl at 110 °C for 90 min. The hydrolysates were lyophilized, suspended in pyridine/acetic acid/water (5:50-945), spotted onto cellulose membrane sheets together with phosphoamino acid standards, and separated by high voltage electrophoresis (950 V for 1 h), followed by autoradiography.

**Treatments and NE Uptake Assays**—Transfected HTR cells were treated with the vehicle or \(\beta\)-PMA (0.5 \mu M) or SP (0.25 \mu M) or \(\beta\)-PMA plus SP for 30 min at 37 °C. Other treatments with various reagents were performed where indicated. Uptake measurements were performed by incubating the cells for 10 min at 37 °C with \(^{3}^{\text{H}}\)NE (35.0 Ci/mmol 1-[7,8-\text{H}]norepinephrine; Amersham Biosciences) in 0.5 ml of Krebs-Ringer-HEPES (KRH) buffer, pH 7.4 (120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl\(_{2}\), 10 mM HEPES, 1.2 mM MgSO\(_{4}\), 1.2 mM KH\(_{2}\)PO\(_{4}\), 5 mM Tris, and 10 mM D-glucose), containing 100 \mu M ascorbic acid and 100 \mu M pargyline. Assays were terminated by removing the radiolabel and by rapid washings of cells three times with 1 ml of ice-cold KRH buffer. Cells were solubilized in 0.5 ml of 1% SDS, and the accumulated \(^{3}^{\text{H}}\)NE was quantified by liquid scintillation counting (Beckman Coulter Inc.). 50 nm of \(^{3}^{\text{H}}\)NE was used in all experiments except for kinetic studies, where uptake of NE was measured over a concentration of 0.1
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to 10 μM NE with 50 nM [3H]NE and the rest substituting with nonradioactive NE. Specific NE uptake was measured by subtracting the NE uptake measured in the presence of 1 μM desipramine from the total NE uptake measured in the absence of desipramine. For kinetic analysis of NE uptake in trophoblasts, the values were plotted as picomoles of NE uptake versus concentration of NE, and the data represent the means ± S.E. from three experiments performed in triplicate on different batches of trophoblast cultures. Substrate \( K_m \) and \( V_{\text{max}} \) values for NE uptake were determined by nonlinear least square fits (Kaleidagraph, Synergy Software, Reading, PA) with the generalized Michaelis-Menten equation:

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 V = \frac{V_{\text{max}} [S]^n}{(K_m + [S])^n}, \]

where \( V \) = transport velocity, \( [S] \) = substrate (NE) concentration, and \( n \) represents the Hill coefficient. Data are represented as the means ± S.E. from three experiments performed in triplicate on different batches of trophoblast cultures.

Cell Surface Protein Biotinylation—Cell surface biotinylation and immunoblot analyses were employed (1) to quantify the amount of plasma membrane NET protein. Transfected cells subjected to various treatments were washed and incubated with the cell membrane-impermeable reagent, sulfosuccinimidobiotin (sulfo-NHS-biotin, 1 mg/ml; Pierce) for 1 h at 4 °C in PBS/Ca-Mg (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 9.6 mM Na2HPO4, 1 mM MgCl2, 0.1 mM CaCl2, pH 7.3). The biotinylating agent was removed by incubating twice with ice-cold 100 mM glycine for 30 min at 4 °C. Cells were washed with PBS/Ca-Mg and lysed at 4 °C with 400 μl of RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) containing protease inhibitors (1 μM pepstatin A, 250 μM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 μg/ml aprotinin). Lysates were centrifuged at 20,000 × g for 30 min at 4 °C, and the supernatants were incubated with monomeric avidin beads for 1 h at room temperature. The beads were washed three times with RIPA buffer, and adsorbed proteins were eluted in 50 μl of Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.01% bromphenol blue). Aliquots from total cell lysates and unbound fractions (40 μl each) and all (50 μl) of the avidin-bound samples were analyzed by immunoblotting with hNET-antibody. To validate the surface localization of biotinylated NET protein, blots were stripped and reprobed with anti-calnexin antibody (1:1000, StressGen Biotechnologies, Victoria, British Columbia, Canada). Band intensities were quantified using NIH ImageJ (version 1.32j). Exposures were precalibrated to ensure quantitation within the linear range of the film, and multiple exposures were taken to validate linearity of quantitation. Values of total, nonbiotinylated, and surface NET proteins were normalized using levels of calnexin immunoreactivity in total cell extract, and values were averaged across three experiments.

NET insertion into the plasma membrane was measured using similar protocols as described before (33) with slight modifications. HTR cells expressing the WT-hNET or the double mutant were washed with PBS/Ca-Mg and incubated twice with 1 mg/ml sulfo-NHS-acetate in PBS/Ca-Mg for 1 h at 4 °C (trafficking nonpermissive condition) to block all the free amino groups (34). After washing away the sulfo-NHS-acetate with cold PBS/Ca-Mg, the cell membrane-impermeable sulfo-NHS-biotin in PBS/Ca-Mg containing β-PMA or the vehicle (1 mg/ml, prewarmed at 37 °C) was added to the cells and incubated further for the indicated times at 37 °C (trafficking permissive condition). Biotinylated NETs inserted into the plasma membrane (surface) and nonbiotinylated (intracellular) NETs were analyzed as described above. Biotinylated transferrin receptor (TfR) was analyzed by stripping and probing the blot with TfR antibody. The accumulation of biotinylated WT-hNET or hNET-DM or TfR at each time point was measured by quantifying the band densities using NIH ImageJ (version 1.32j).

To measure NET internalization, reversible biotinylation assays were performed as described (1). HTR cells expressing the WT-hNET or the double mutant were cooled rapidly to 4 °C to inhibit endocytosis by washing with cold PBS and surface-biotinylated with disulfide-cleavable biotin sulfo succinimidyl-2-(binihamido)ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin, 1 mg/ml; Pierce), and free biotinylating reagent was removed by quenching with glycine. NET endocytosis was initiated by incubating the cells with prewarmed media containing β-PMA or the vehicle for indicated times at 37 °C. At the end of each time point, the reagents were removed, and fresh pre-chilled media were added to stop the endocytosis. The cells were then washed and incubated twice with 250 μM sodium 2-mercaptoethanesulfonate (MesNa; Sigma), a reducing agent, in PBS/Ca-Mg for 20 min to dissociate the biotin from cell surface-resident proteins via disulfide exchange. To define total biotinylated NETs, one dish of biotinylated cells was not subjected to reduction with MesNa and was directly processed for extraction followed by isolation by avidin beads. To define MesNa-accessible NETs, another dish of cells was treated with MesNa immediately (at 0 time) following biotinylation at 4 °C to reveal the quantity of surface NET biotinylation that MesNa can reverse efficiently. At the end of the treatments, cells were solubilized in RIPA, and biotinylated NETs were separated from nonbiotinylated proteins by using monomeric avidin beads. Biotinylated proteins were eluted from the beads and resolved by SDS-PAGE. NET proteins in the fractions were visualized with hNET antibody as described under “Cell Surface Protein Biotinylation.” NET bands were scanned and the band densities were quantified by NIH ImageJ (version 1.32j).

Lipid Raft Isolation—Lipid rafts were isolated from vehicle- or β-PMA-treated cells essentially using the same protocol described earlier (1, 35). Cells were lysed in 1.5 ml of MBS (25 mM Mes and 150 mM NaCl, pH 6.5) containing 1% Triton X-100 and a mixture of protease inhibitors (1 μM pepstatin A, 250 μM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 μg/ml aprotinin) using a Dounce homogenizer with 10 up and down strokes at 4 °C. Equal volumes of 80% (w/v) sucrose in MBS were added to the homogenates. Cell lysates in 40% sucrose were placed at the bottom of SW41 centrifuge tubes and overlaid successively with 4 ml of 30% sucrose and 3 ml of 5% sucrose. The tubes were centrifuged at 188,000 × g for 18 h at 4 °C, and 1-ml fractions were collected from the top. Equal volumes of collected fractions were subjected to 4–15% linear gradient SDS-PAGE. After transfer to polyvinylidene difluoride
Immobilon-P transfer membrane, the presence of NET and caveolin were visualized by immunoblotting with specific antibodies.

**FIGURE 1. Phosphoamino acid analysis.** Rat placental trophoblasts (T) and HTR cells transfected with WT-hNET (H) were metabolically 32P-labeled and treated with β-PMA (0.5 μM) for 30 min. 32P-labeled NETs were immunoprecipitated using NET-specific antibody and subjected to phosphoamino acid analysis as described under “Experimental Procedures.”

**FIGURE 2. Schematic representation of the human norepinephrine transporter.** Analysis of hNET amino acid sequence by NetPhos 2.0 program revealed serine 259 as a potential consensus PKC site and threonine 258 as a nonconsensus site. Large black circles with white letters indicate mutated serine and threonine residues at the predicted PKC site. Large gray circles with white letters indicate potential phosphorylation sites for other kinases.

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**Metabolic Labeling and Immunoprecipitation (Phosphorylation Assay)—**Transfected cells were incubated at 37 °C in phosphate-free DMEM for 1 h and then with 1 mCi/ml of carrier-free [32P]orthophosphate (Amersham Biosciences) for 2 h (1). The vehicle or β-PMA (0.5 μM) or SP (0.25 μM) was added to the medium, and the incubation was continued at 37 °C for 30 min. Cells were washed with cold PBS and lysed in 400 μl of RIPA buffer containing protease inhibitors (1 μM peptatin A, 250 μM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 μg/ml aprotinin) and phosphatase inhibitors (10 mM NaF, 50 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1 μM of okadaic acid). Extracts were centrifuged at 20,000 × g for 30 min at 4 °C, and the supernatants were precleared using 50 μl of protein G-Sepharose beads (36). NET protein was immunoprecipitated overnight at 4 °C by the addition of anti-His antibody to specifically isolate His-tagged NET proteins and to avoid interference with any endogenous NET if present on endover-end continuous mixing, followed by 1.5-h incubation with protein G-Sepharose beads (50 μl) at 22 °C (room temperature). The immunoadsorbents were washed with ice-cold RIPA buffer, extracted with 50 μl of Laemmli, and resolved by SDS-PAGE (10%). The radiolabeled proteins were detected by autoradiography, and digitized autoradiograms were evaluated on multiple film exposures to ensure quantitation within the linear range of film exposure. In parallel experiments, immunopre-
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cipitations were carried out using unlabeled cells to ensure that equal NET proteins were immunoprecipitated. Extracts from each sample were immunoprecipitated with anti-His antibody, and the immunosolanes were subjected to urea-based SDS-PAGE and transferred on to polyvinylidene difluoride membranes. NET proteins were quantified by immunoblotting with hNET antibody.

In Vitro Phosphorylation Assay—In vitro phosphorylation with PKCe was performed using membranes prepared from HTR cells expressing the WT, single-site (T258A and S259A) and double-site mutant hNETs. Membranes were prepared as described previously (1, 37) and washed twice with PKC assay buffer (20 mM HEPES, pH 7.4, 10 mM MgCl₂, and 1 mM dithiothreitol). In a total 100-μl PKC assay buffer, 20 μg of membrane proteins (resuspended in PKC assay buffer) were incubated at 30 °C for 30 min with 20 μCi of [γ-32P]ATP (Amersham Biosciences), 100 μM ATP, 200 μg/ml phosphatidylserine, and 20 μg/ml diacylglycerol in the presence or absence of PKC (1 μl).

Following phosphorylation, the membranes were solubilized in RIPA buffer containing protease and phosphatase inhibitors, and immunoisolated NET proteins were analyzed by SDS-PAGE and autoradiography as described above. To ensure equal immunoisolation of NET protein between control and test samples (−PKCe and +PKCe), parallel immunoprecipitations and immunoblot analyses were carried out using membranes subjected to phosphorylation but without the addition of [γ-32P]ATP.

Statistical Analyses—Statistical significances for NE uptake values and band densities were calculated using Student’s t test when comparisons were made between two groups (each treatment compared with respective vehicle control). Analysis by one-way analysis of variance (ANOVA) was used followed by post hoc testing (Bonferroni) when comparisons were made between more than two groups.

RESULTS

PKC Activation Induces Phosphorylation of NET on Serine and Threonine Residues—Recently, we have demonstrated that PKC activation by β-PMA induces phosphorylation of native NETs in rat placental trophoblasts (1). Similarly, we observed enhanced phosphorylation of WT-hNET expressed in HTR cells following β-PMA treatment. To identify the type of residues that are phosphorylated on endogenous NETs and heterologously expressed hNET, we performed phosphoamino acid analysis on immunoisolated 32P-labeled NETs. Immunosolanes were carried out essentially as described before (1). Phosphoamino acid analysis of 32P-labeled NETs from metabolically labeled rat placental trophoblasts and HTR cells expressing the WT-hNET revealed the presence of phosphoserine and phosphothreonine residues following β-PMA treatment (Fig. 1). Low levels of phosphoserine and phosphothreonine residues were observed in vehicle-treated cells corresponding to basal NET phosphorylation. These results indicate that PKC activation leads to phosphorylation of both serine and threonine residues in native rat NET as well as in heterologously expressed hNET.

Mutation of the PKC Motif in hNET Attenuates β-PMA-mediated NE Uptake Inhibition—Analysis of hNET amino acid sequence (Fig. 2) by NetPhos 2.0 program revealed Ser-259 as a potential consensus PKC site and Thr-258 as a nonconsensus site. Based on this analysis and the results from phosphoamino acid analysis (Fig. 1), we generated three mutants as follows: two single-site mutants and a double mutant at this predicted PKC motif. HTR cells transiently transfected with the WT-hNET and the PKC site mutants of hNET were analyzed for expression levels and NE uptake capacities. The results are shown in Fig. 3, A and B, respectively. T258A and S259A single-site mutants were expressed at levels similar to those of WT-hNET and exhibited 90–100% of wild type NE uptake capacity (Fig. 3, A and B). However, the T258A/S259A double mutant was expressed at about 50% of WT-hNET level and exhibited about 50% of wild type NE uptake capacity when measured at single (50 nM) substrate (NE) concentration (Vο). The mutant transporters were then examined for their sensitivity to PKC activation. As shown in Fig. 3C, HTR cells expressing the WT-
hNET displayed a strong (~50%) inhibition of NE uptake in response to 0.5 μM β-PMA. In striking contrast, HTR cells expressing the double mutant (T258A/S259A) displayed no inhibition. Interestingly, compared with a 50% inhibition of WT-hNET and hNK-1 receptor with the PKC activator, Phorbol 12-myristate 13-acetate (PMA), decreased NE uptake in a dose- and time-dependent manner that was similar to our earlier findings with rat trophoblasts expressing native NETs (1). In HTR cells expressing the WT-hNET and hNK-1 receptor, a maximum inhibition of 50% of NE uptake was observed after a 30-min incubation with 0.5 μM β-PMA (Fig. 4A). Activation of hNK-1 receptor coexpressed with WT-hNET in HTR cells by 0.25 μM SP inhibited 30% of NE uptake after 30 min of incubation (Fig. 4A). Treatment of HTR cells expressing the hNET and hNK-1 receptor with β-PMA and SP together did not result in a higher inhibition than the 50% inhibition observed with β-PMA treatment alone (Fig. 4A). In addition, staurosporine, the broad spectrum kinase inhibitor, completely blocked the inhibitory effect of β-PMA and SP suggesting that the activation of PKC is involved in β-PMA- or SP-induced decreases in NE uptake (Fig. 4A). In HTR cells expressing the hNET double mutant and hNK-1 receptor, β-PMA or SP or β-PMA plus SP or staurosporine had no significant effect on NE uptake (Fig. 4B). Because our immunoblot analyses revealed that the double mutant is expressed at about 50% of WT-hNET levels (Fig. 3A), we have reexamined the effects of β-PMA and SP by doubling the hNET double mutant cDNA to normalize the expression levels. Under these conditions, both β-PMA and SP failed to inhibit the double mutant suggesting that the absence of regulation by PKC activation is not because of changes in expression levels (Fig. 4C).

To assess the mechanism of the inhibitory effect of PKC activation, saturation uptake experiments were carried out with WT-hNET and the double mutant following a 30-min treatment with β-PMA or vehicle. Kinetic analysis revealed that β-PMA (0.5 μM) treatment of HTR cells expressing the WT-hNET decreased the maximal velocity (V_{max}) by ~50% (vehicle, 373.73 ± 14.02 pmol/100,000 cells/10 min; β-PMA, 178.55 ± 3.47 pmol/100,000 cells/10 min) but did not alter the K_{m} value for NE uptake (K_{m} values: vehicle control, 4.24 ± 0.49 μM; β-PMA, 4.28 ± 0.26 μM) (Fig. 4B).

The hNET Double Mutant Is Completely Resistant to PKC-mediated Sequestration from the Plasma Membrane—Biotinylation experiments were performed to assess changes in surface NET following β-PMA or SP treatment of HTR cells transfected with WT-hNET plus hNK-1 receptor or hNET double mutant plus hNK-1 receptor. As shown in Fig. 6A, in cells expressing WT hNET, there was a significant decrease in the amount of immunoreactive NET proteins that were surface-biotinylated following treatments with β-PMA or SP. β-PMA or SP treatment decreased NET immunoreactivity by 35–40% of vehicle control value in the biotinylated or cell surface fraction (Fig. 6A). Corresponding increases in the amount of nonbiotinylated intracellular transporter were observed in β-PMA- or SP-treated cells (Fig. 6A). In contrast, neither β-PMA nor SP had a significant effect on the surface expression of hNET double mutant (Fig. 6A). This result is consistent with the β-PMA or SP effect on NE transport in HTR cells expressing the double mutant and suggested that the double mutant is indeed completely resistant to PKC-mediated down-regulation of hNET. In both groups (WT and the double mutant), there were no significant differences in total NET protein levels between vehicle or β-PMA or SP treatments (Fig. 6A). Results from surface biotinylation experiments revealed no changes in the ratio of surface versus total NET protein expression between WT (1:3.8) and the double mutant (1:3.6) (Fig. 6) suggesting that the mutation does not

**FIGURE 4.** hNET double mutant is resistant to β-PMA- and SP-induced inhibition mediated by PKC activation. HTR cells transfected with hNK-1 receptor plus the WT-hNET (A) or hNK-1 receptor plus the double mutant (B) were treated with β-PMA (0.5 μM) or SP (0.25 μM) or β-PMA plus SP or staurosporine (Staur, 1 μM) alone or pretreated with 1 μM staurosporine for 20 min before β-PMA plus SP treatment for 30 min. NE uptake assays were performed as described under “Experimental Procedures.” C HTR cells transfected with hNK-1 receptor plus the double mutant (0.5 μg of cDNA instead of 0.25 μg) were used for NE uptake measurements following treatment with β-PMA (0.5 μM) or SP (0.25 μM). Data derived from three separate experiments, each in triplicate, are given as mean ± S.E. Values were significantly different from vehicle control (*, p < 0.01, one-way ANOVA with Bonferroni post hoc analysis).

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change the distribution of hNET double mutant (85-kDa species) between the surface and intracellular pools. However, there was more of nonglycosylated 48-kDa NET protein in the double mutant compared with that of WT-hNET, which was found mostly in the intracellular pool. Quantified band densities of biotinylated hNET (85-kDa bands) expressed in percent of total NET are presented in Fig. 6B. When reprobed with anti-calnexin antibody, no differences in the calnexin levels were observed in the total fractions between treatments suggesting equal loading and transfer of proteins, and also calnexin shows no differences between total fractions and very little calnexin in the bound fractions. B, bar graph shows biotinylated hNET (85 kDa) expressed in percent of total. Results from three independent experiments are given as mean ± S.E. * indicates significant difference in the V_max values for β-PMA-treated cells compared with vehicle-treated cells (p < 0.01, Student’s t test).
was not detected in the bound fractions suggesting no contamination with intracellular proteins (Fig. 6A).

The Plasma Membrane Delivery of WT-hNET and hNET Double Mutant Is Altered Similarly by PKC Activation—A decrease in the plasma membrane expression level of WT-hNET following PKC activation (β-PMA treatment) could arise from either an inhibition in the plasma membrane insertion or an increase in the endocytosed NET. Fig. 7 shows the results from biotinylation experiments performed to measure the plasma membrane insertion of WT-hNET and the double mutant. In HTR cells expressing the WT-hNET or the double mutant, there was a time-dependent increase in the biotinylated transporters with a concomitant disappearance of intracellular transporters following vehicle treatment (Fig. 7, A and B). There was no biotinylated NET (WT or the double mutant) at the zero time point in sulfo-NHS-acetate-treated cells, suggesting that all pre-existing surface NETs are completely blocked (from modification by biotinylation) and thus that biotinylated NET observed in subsequent time points after warming the cells to 37 °C represents newly delivered NET only. These results suggested that hNET is constitutively inserted into the plasma membrane, and this constitutive insertion is not altered by T258A/S259A double mutation. Interestingly, there were also no significant changes in the plasma membrane insertion of WT-hNET or the double mutant following β-PMA treatment (Fig. 7, A and B). Time-dependent increases in plasma membrane TIR levels were observed under similar conditions indicating that under trafficking-permissive conditions a significant amount of NET (WT or the double mutant) reaches the plasma membrane in 30 min (Fig. 7). β-PMA had no effect on TIR insertion. The time course of plasma membrane insertion of WT-hNET or hNET double mutant or TIR was fitted (fits not shown) to a single exponential equation as follows: \( A(1 - \exp(-t/\tau)) \), where \( A \) stands for the maximum band density; \( \tau \) stands for time (in minutes), and \( \tau \) stands for the time constant of the process, the inverse of which is the exocytosis rate. Using fits to this equation, the time constants \( \tau \) were found to be 13.0 ± 0.8 min for WT-hNET, 15.7 ± 0.4 min for the double mutant, and 3.8 ± 0.8 min for TIR. The exocytosis rates of WT-hNET, hNET-DM, and TIR were 0.0767 ± 0.0045, 0.0636 ± 0.0015, and 0.2619 ± 0.0573 min⁻¹, respectively. There were essentially no changes in the exocytosis rates of WT-hNET or the double mutant or TIR following β-PMA treatment (Fig. 7). Next, we sought to examine the internalization of both WT-hNET and the double mutant under basal/constitutive and PKC-stimulated conditions by using reversible biotinylation strategies and by quantifying the fraction of surface NET that moves in a time-dependent manner to an intracellular compartment. Biotin from biotinylated proteins remaining on the surface at the end of a particular treatment protocol was removed by treatment with MesNa, a nonpermeant reducing agent that reduces disulfide bonds and liberates biotin from biotinylated proteins at the cell surface. The amount of biotinylated proteins resistant (inaccessible) to MesNa treatment or reversal of biotinylation is defined as “the amount of protein endocytosed or internalized.” Incubation of the cells at 37 °C in the absence or presence of β-PMA before MesNa reversal of surface biotinylation permitted evaluation of NET internalization occurring in the absence of β-PMA (basal endocytosis) versus β-PMA-mediated changes in NET internalization (β-PMA-stimulated endocytosis). The 1st lane in Fig. 8, A and B, shows the amount of total NET that is biotinylated (no MesNa treatment). MesNa treatment immediately after biotinylation showed less than 0.5% of biotinylated NET (Fig. 8, A and B, 2nd lane) indicating very little internalization at 4 °C. Following treatment with vehicle alone, a gradual (5–30%) increase in biotinylated NET was seen over time (5–30 min) in cells expressing the WT-hNET or the double mutant (Fig. 8, A and B). This increase in the internalized NET represents constitutive or basal endocytosis. As shown in the Fig. 8, A and B, there was no significant difference in the constitutive endocytosis between WT-hNET and the double mutant. Treatment with β-PMA resulted in a significant (2-fold) increase in the amount of WT-hNET internalized compared with vehicle treatment at all (5, 15, and 30 min) time periods examined (Fig. 8A). Under similar conditions, β-PMA failed to increase the amount of hNET double mutant internalized. The percent internalization of WT-hNET and the double mutant are shown in the lower panels of Fig. 8, A and B. In the WT-hNET, ~5% of surface-biotinylated fraction was internalized by 5 min under unstimulated (basal) conditions. This was increased to ~10% (2-fold increase) following β-PMA treatment. Similar increases were observed in the amount of WT-hNET internalization following 15-min (~20% in vehicle versus ~40% in β-PMA) and 30-min (~30% in vehicle versus ~60%) treatments. In cells expressing the double mutant, there were no significant differences in the amount of NET internalization following β-PMA treatment compared with vehicle treatment (~5% at 5
PKC-mediated NET Phosphorylation Is Significantly Blunted in the hNET Double Mutant—To examine whether the hNET double mutant that is resistant to PKC-mediated down-regulation undergoes phosphorylation following β-PMA treatment, we performed phosphorylation assays using HTR cells transfected with WT-hNET (A) or hNET double mutant (B) were treated with β-PMA (0.5 μM) or the vehicle for the indicated times. Following MesNa treatment, the cells were solubilized in MBS containing 1% Triton X-100 and subjected to sucrose gradient centrifugation as described under "Experimental Procedures." Representative immunoblots of three separate experiments are shown. Phosphorylation-dependent Internalization of NET mediated redistribution from rafts.

PKC-mediated NET Phosphorylation Is Significantly Blunted in the hNET Double Mutant—To examine whether the hNET double mutant that is resistant to PKC-mediated down-regulation undergoes phosphorylation following β-PMA or SP treatment, we performed phosphorylation assays using HTR cells

min, ~20% at 15 min, and ~30% at 30 min). These results indicate that Thr-258 and Ser-259 residues control PKC-mediated endocytosis but not constitutive internalization.

The hNET Double Mutant Is Resistant to β-PMA-mediated Redistribution from Lipid Rafts—Recently, we have demonstrated that PKC-mediated NET internalization is not mediated by dynamin-dependent, clathrin-mediated endocytic pathway but via lipid rafts (1). Because hNET double mutant was resistant to PKC-mediated effects, including sequestration from the plasma membrane, we examined whether hNET double mutant is localized in lipid rafts and whether raft-associated mutant hNET redistributes from rafts upon PKC activation. Discontinuous sucrose gradient centrifugation was used to measure the amount of WT-hNET and hNET double mutant present in the lipid raft and nonlipid raft fractions following vehicle or β-PMA treatment. Immunoblot analyses of the proteins in the isolated fractions revealed that hNET proteins are present in lipid raft fractions (fractions 3–5) (Fig. 9, A and B). Caveolin, a marker for lipid rafts, was specifically detected in lipid raft fractions (Fig. 9, A and B). Treatment of HTR cells expressing the WT hNET with β-PMA decreased the levels of NET in the lipid raft fractions, with concomitant increases of NET protein in the non-lipid raft fractions (Fig. 9A). The loss of NET from the raft fraction (Fig. 9) appears to be greater than the decrease in cell surface NET following PKC stimulation (Fig. 6). It is possible that only part of the raft-associated NET might be internalized. However, the differences in the methods of analysis (raft isolation versus biotinylation experiments) could not be ruled out. Nonetheless, upon β-PMA treatment, there was no change in the levels of T258A/S259A hNET double mutant in the lipid raft or nonlipid raft fractions (Fig. 9B). Together, these results indicate the involvement of lipid rafts in the PKC-mediated internalization of WT-hNET and provide evidence that the hNET double mutant is resistant to PKC-mediated redistribution from rafts.
transfected with hNK-1 receptor plus WT-hNET or the double mutant. Here, we have increased (doubled) hNET double mutant cDNA in the transfections to match WT-hNET expression level. The cells were labeled with [32P]orthophosphate before stimulation with 0.5 μM β-PMA or 0.25 μM SP. The transporter was immunoprecipitated from the cell extracts with anti-His antibody and analyzed by SDS-PAGE and autoradiography. The phospho-hNET protein bands at ~85 kDa corresponding to the mature, fully glycosylated transporter are shown in Fig. 10A, lower panel. Less intense phosphorylated bands observed in the vehicle-treated cells (Fig. 10A, lower panel, 1st and 4th lanes) represent basal NET phosphorylation, and the basal phosphorylation of hNET double mutant appeared to be higher compared with that of WT-hNET when corrected for NET protein levels. Treatment of metabolically labeled HTR cells expressing the hNK-1 receptor plus WT-hNET with 0.5 μM β-PMA or 0.25 μM SP resulted in a 2.5-fold increase in the phosphorylation of hNET protein (Fig. 10A, 2nd and 3rd lanes) when compared with vehicle-treated control (Fig. 10A, 1st lane). However, there was only about 1.5-fold increase in the phosphorylation of double mutant following 0.5 μM β-PMA or 0.25 μM SP treatments (Fig. 10, 5th and 6th lanes) compared with vehicle-treated control (Fig. 10A, 4th lane). The decrease in the stimulation of phosphorylation of the hNET double mutant is not because of lower expression levels because similar results were observed when we equalized the expression of hNET double mutant to that of WT-hNET levels (Fig. 11B). Together, these results indicated that the PKC-induced phosphorylation of hNET double mutant was significantly (~40%) less compared with that of WT-hNET. The phospho-hNET bands were not visible from the immunoprecipitates isolated from HTR cells transfected with the hNK-1 receptor plus the vector. Parallel immunoprecipitations and Western blot analysis of the hNETs from unlabeled cells showed similar quantities of WT-hNET and hNET double mutant proteins in vehicle-, β-PMA-, and SP-treated cells (Fig. 10A, upper panel). This way, we verified that the changes observed in the phosphorylation state of the double mutant or the WT-hNET were not because of discrepancies in our immunoprecipitation procedures. Quantitative results from averaged values from three separate phosphorylation experiments indicate that the PKC-mediated phosphorylation of the hNET double mutant is significantly blunted (Fig. 10, B and C). Taken together, these data suggest that the amino acids Thr-258 and Ser-259 represent a target PKC motif in hNET and that substitution of these residues with nonphosphorylatable alanines renders the hNET protein less susceptible to be phosphorylated in response to PKC activation.

**PKC-mediated Phosphorylation but Not Sequestration Is Impaired in the Single Mutants**—Because the T258A single-site mutant exhibited partial resistance to inhibition by β-PMA, we examined the changes in the plasma membrane levels and the phosphorylation of T258A and S259A single-site mutants following β-PMA treatment. Although the double mutant is expressed at about half the level of WT-hNET, the expression levels of T258A or S259A single mutants were similar to WT-hNET expression (Fig. 3A). Therefore, we equalized the expression level of the double mutant by

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**FIGURE 10. β-PMA- and SP-induced phosphorylation of hNET is blunted in HTR cells expressing the hNK-1 receptor plus hNET double mutant.** A, HTR cells transfected with hNK-1 receptor cDNA (0.5 μg) plus WT-hNET cDNA (0.5 μg) plus vector (0.5 μg) or hNK-1 receptor cDNA (0.5 μg) plus hNET double mutant cDNA (1.0 μg) were metabolically labeled with [32P]orthophosphate. Metabolically labeled cells were treated with vehicle, or β-PMA (0.5 μM), or SP (0.25 μM) and subjected to immunoprecipitations with anti-His antibody followed by autoradiography as described under “Experimental Procedures.” Phosphorylated protein bands corresponding to hNET protein are shown in the lower panel. Parallel immunoprecipitations were performed using unlabeled cells, and the immunoprecipitates were subjected to urea-based SDS-PAGE and immunoblotting with hNET antibody. A representative immunoblot is shown in the upper panel. Phosphorylation of WT-hNET and the double mutant (represented as [32P]hNET/hNET ratios) from three independent experiments are given as mean values ± S.E. B, asterisks indicate significant increases in [32P] labeling of NET by β-PMA or SP treatment compared with vehicle-treated control (*, p < 0.01; **, p < 0.05, Student’s t test and one-way ANOVA with Bonferroni post hoc analysis). C, shows PKC-mediated stimulation of hNET phosphorylation. * indicates significant decrease in [32P] or SP-induced phosphorylation of hNET double mutant compared with that of WT-hNET (p < 0.01, Student’s t test and one-way ANOVA with Bonferroni post hoc analysis).
increasing the double mutant cDNA to 2-fold (1 μg) compared with 0.5 μg of WT-hNET or single-site mutant cDNAs in our transfections, and we examined the loss of plasma membrane hNET levels using surface biotinylation experiments and NET phosphorylation using metabolic labeling. The results are shown in Fig. 11. In cells expressing the WT-hNET or T258A or S259A mutant hNET’s, there was a significant (~45%) decrease in the amount of immunoreactive NET proteins that were surface-biotinylated following β-PMA treatment (Fig. 11). There was no change in the biotinylated hNET level in cells expressing the double mutant following β-PMA treatment (Fig. 11A). These results further confirmed our findings that the double mutants but not the single-site (T258A or S259A) mutants are completely resistant to PKC-induced down-regulation. The results from phosphorylation experiments are shown in Fig. 11B. Interestingly, there was a significant increase (1.6–1.7-fold) in the basal (vehicle treatment) phosphorylation of T258A and S259A single-site mutants as well as the double mutant compared with WT-hNET basal phosphorylation. However, β-PMA induced about 1.5–1.6-fold increase in the basal phosphorylation of the double mutant and T258A single-site mutant compared with a 3.2-fold increase in the WT-hNET basal phosphorylation. This represented a 50% block of β-PMA effect on T258A or the double mutant phosphorylation. Interestingly, β-PMA completely failed to stimulate phosphorylation of S259A (Fig. 11B). Although the PKC-mediated phosphorylation was either completely or partially blunted in the single-site mutants, plasma membrane sequestration was not affected as evidenced by biotinylation experiments (Fig. 11A). Together, these results suggest that individual phosphorylation of either Thr-258 or Ser-259 alone has no influence on transporter internalization.

Alanine Substitution of All Other Potential Phosphosites in the Absence of T258A and S259A Double Mutation Does Not Prevent PKC-induced hNET Phosphorylation and Down-regulation—Although PKC-mediated down-regulation was completely abolished in the double mutant, PKC-induced phosphorylation was partially abrogated. It is possible that other serine and threonine residues identified as potential phosphorylation sites (NetPhos analysis) may be involved in restoring some of the PKC-mediated phosphorylation. To test such a possibility, we have substituted serine and threonine residues as indicated under the “Experimental Procedures” with alamines in the presence and absence of T258A plus S259A double mutation and examined the effect of β-PMA on NE uptake, NET sequestration, and phosphorylation. The mutant hNET with all potential phosphorylation sites replaced with alamines except for Thr-258 and Ser-259 is represented as N’ + C’ + S502A mutant. The mutant hNET with all potential phosphorylation sites replaced with alamines, including Thr-258 and Ser-259, is represented as N’ + C’ + S502A + DM. N’ + C’ + S502A mutant exhibited similar PKC sensitivity as that of WT-hNET with respect to NET down-regulation and phosphorylation (Fig. 12). β-PMA induced a 50% inhibition of NE uptake by WT-hNET and N’ + C’ + S502A mutant and completely failed to inhibit NE uptake by N’ + C’ + S502A + DM (Fig. 12A). Although N’ + C’ + S502A mutant was expressed at 50–60% of WT-hNET level, N’ + C’ + S502A + DM was expressed at about 25% of WT-hNET level. Surface biotinylation analysis revealed a 40–45% loss of plasma membrane expression of WT-hNET and N’ + C’ + S502A mutant following β-PMA treatment (Fig. 12B). Under identical conditions, the surface expression of N’ + C’ + S502A + DM was not changed (Fig. 12B). These results suggested that T258A plus S259A but not other potential kinase sites control PKC-mediated NET endocytosis. Analysis of hNET phosphorylation indicated...
that following β-PMA treatment, there was a 3.5–3.6-fold stimulation in the basal phosphorylation of WT-hNET (Fig. 12C). To our surprise, N’ + C’ + S502A mutant, lacking all potential phosphorylation sites except for Thr-258 and Ser-259, was also found in phosphorylated form like WT-hNET, and the basal phosphorylation of hNET double mutant (3.2-fold stimulation) in the phosphorylation assays. As shown in Fig. 13, a small but significant amount of NET (WT as well as mutants) was phosphorylated in the absence of external addition of PKCe (Fig. 13, 1st, 3rd, 5th, and 7th lanes), and the basal phosphorylation of hNET mutants appeared to be higher compared with that of WT-hNET when corrected for NET protein levels. Exogenous addition of PKCe resulted in a significant increase (3.2-fold stimulation) in the phosphorylation of WT-hNET (Fig. 13, 2nd lane). Under similar conditions, addition of PKCe resulted in about 1.5–1.6-fold increase in the phosphorylation of hNET double mutant and T258A mutant (Fig. 13, 4th and 6th lanes), a significant (~55%) reduction in the phosphorylation of these mutants compared with WT-hNET phosphorylation. Interestingly, exogenous addition of PKCe preparations (to maintain native conformation of NET) containing WT and single and double mutant hNETs. Based on our previous observation that a calcium-independent PKC isoform, most probably PKCe, may be involved in NET phosphorylation in the placental trophoblasts (1), we have used purified PKCe in in vitro phosphorylation assays. As shown in Fig. 13, a small but significant amount of NET (WT as well as mutants) was phosphorylated in the absence of external addition of PKCe (Fig. 13, 1st, 3rd, 5th, and 7th lanes), and the basal phosphorylation of hNET mutants appeared to be higher compared with that of WT-hNET when corrected for NET protein levels. Exogenous addition of PKCe resulted in a significant increase (3.2-fold stimulation) in the phosphorylation of WT-hNET (Fig. 13, 2nd lane). Under similar conditions, addition of PKCe resulted in about 1.5–1.6-fold increase in the phosphorylation of hNET double mutant and T258A mutant (Fig. 13, 4th and 6th lanes), a significant (~55%) reduction in the phosphorylation of these mutants compared with WT-hNET phosphorylation. Interestingly, exogenous addition of PKCe

**DISCUSSION**

Recent reports demonstrated that monoamine transporters are sequestered from the plasma membrane in response to PKC activation via several mechanisms (1, 38), and transporter down-regulation occurs parallel to an increase in transporter protein phosphorylation (1, 36, 39). In this study, we show that the PKC activation induces phosphorylation of both serine and threonine residues in rat and human NETs, and we demonstrate a possible link between transporter phosphorylation and down-regulation. Phosphorylation of serine and threonine residues has been described for DAT (28, 40). SERT and GAT1 are also phosphorylated in response to PKC activation (36, 41), and PKC-mediated phosphorylation of SERT occurs initially on serine residues followed by threonines and is associated with
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FIGURE 13. hNET is phosphorylated in vitro by exogenous PKCε and is blunted by S259A mutation. In vitro phosphorylation assays were carried out on membranes prepared from HTR cells transfected with WT-hNET (0.5 µg) or hNET double mutant (0.5 µg) or T258A mutant (0.5 µg) or S259A mutant (0.5 µg) as described under “Experimental Procedures.” A, phosphorylated protein bands corresponding to hNET protein are shown in the lower panel. Parallel immunoprecipitations were performed using nonphosphorylated membranes, and the immunoprecipitates were subjected to urea-based SDS-PAGE and immunoblotting with hNET antibody. A representative immunoblot is shown in the upper panel. Phosphorylation of WT-hNET and the hNET mutants (represented as 32P-hNET/hNET ratios) from three independent experiments are given as means ± S.E. B, asterisks indicate significant increase in 32P-labeling of NET in the presence of PKCe compared with that in the absence of PKCe (*, p < 0.01; **, p < 0.05, Student’s t test and one-way ANOVA with Bonferroni post hoc analysis). Carets indicate significant increase in the basal phosphorylation of hNET mutants compared with WT-hNET basal phosphorylation (p < 0.05, Student’s t test and one-way ANOVA with Bonferroni post hoc analysis).

WT-hNET basal phosphorylation (p < 0.05, Student’s t test and one-way ANOVA with Bonferroni post hoc analysis). C shows PKCe stimulated phosphorylation. Asterisks indicate significant decrease in the PKCe-stimulated phosphorylation of hNET mutants compared with that of WT-hNET (*, p < 0.01; **, p < 0.05, Student’s t test and one-way ANOVA with Bonferroni post hoc analysis).

Transporter down-regulation (42). Mutation of multiple serines and threonines, alone or in combination, indicated no relationship between DAT serine/threonine phosphorylation and DAT regulation (28, 29). A reciprocal relationship exists between PKC- and tyrosine kinase-mediated GAT1 phosphorylation, and a balance between these two states of phosphorylation may dictate relative abundance of GAT1 on the cell surface (27, 43). The hNET mutant, harboring T258A but not S259A, displays partial resistance to PKC-mediated inhibition. However, hNET double mutant harboring both T258A and S259A was completely resistant to PKC-mediated inhibition. The absence of PKC-mediated regulation of the hNET double mutant is not because of changes in the expression levels because we have reexamined our findings by adjusting the hNET cDNAs to normalize the expression levels. The hNET double mutant is expressed at about half the WT-hNET level. Conceivably, this may arise because of an altered rate of biosynthesis and/or degradation of the mutant transporter. DAT mutants, which displayed lack of phosphorylation, were also expressed at lower levels than the WT-DAT (28). The hNET double mutant displayed lower $K_m$ and $V_{max}$ values compared with WT-hNET. Phospho-site mutations could contribute to altered NE binding and transport processes because of physical conformational changes occurring as the result of mutation or altered phosphorylation state itself. Interestingly, recent studies on DAT harboring mutations in the second intracellular loop (ICL2) reported altered ligand-binding properties attributed to structural changes in the physical conformation of DAT protein (44–46). The structural basis of T258A/Ser-259 mutation remains to be explored. Studies on NET mutants have documented a critical contribution of both N- and C-terminal domains in transporter expression, trafficking, stability and functional regulation (47, 48).

hNET double mutant exhibited normal constitutive recycling like that of WT-hNET and β-PMA had no effect on plasma membrane insertion of the WT-hNET or the double mutant. These observations suggest that Thr-258/Ser-259 motif is not required for constitutive or PKC-independent NET recycling/insertion. GAT1 internalization but not recycling is PKC-dependent (49). The plasma membrane insertion or exocytosis rates of WT-hNET and the double mutant are about 10 times slower than that reported for GAT1 (49). Our calculated exocytosis rate of TIR is about two times slower than that reported in adipocytes (50). To our knowledge, the exocytosis rate of NET has not been reported. The difference between NET and GAT1 exocytosis rates could be explained as transporter-specific or cell-specific. DAT internalization and recycling are modulated by PKC activation but in differentially saturable levels (51). T258A plus S259A double mutant exhibited normal constitutive endocytosis like that of WT-hNET. In agreement with our previous observation (1), β-PMA increased WT-hNET internalization but failed to alter hNET double mutant endocytosis, indicating that Thr-258/Ser-259 motif is required for
PKC-induced internalization. Nonclassical endocytic signals dictate constitutive and PKC-regulated internalization of DAT (52, 53). These signals are conserved in NET. Thus, multiple factors/motifs may dictate endocytosis versus recycling, and distinct mechanisms may be involved in determining constitutive and PKC-regulated transporter trafficking. Following PKC activation, DAT is directed to lysosomal degradation (38), and a recent report suggests that PKC-induced DAT ubiquitination may target DAT to lysosomal degradation (54). Whether PKC induces ubiquitination of NET and whether internalized NET is targeted to degradation or recycling remains to be explored. Although the localization of hNET in lipid rafts was not altered in the double mutant, the β-PMA-mediated redistribution is blocked. These results suggest that the PKC motif is not required for transporter association with the rafts, but in turn may serve as a cellular (phosphorylation) signal required for transporter redistribution from the rafts. The spatial segregation of transporter proteins in raft and nonraft membrane domains suggest that signaling machinery specific to lipid rafts may provide a mechanism linking receptors, kinase(s), and phosphatases to transport regulation. Interestingly, it has been shown that the NK-1 receptor localizes in lipid rafts and PKC relocates to rafts upon NK-1 receptor activation (55).

Although Ser-259 appears to be the site for direct action of PKCα, Ser-259 phosphorylation is not required for PKC-mediated NET down-regulation. T258A mutation partly blunted both PKC-mediated phosphorylation and down-regulation (activity only). However, the fact that the double mutation partially eliminates PKC-induced phosphorylation and completely blocks NET down-regulation/internalization suggests a possible relationship between Thr-258/Ser-259 motif phosphorylation and transporter internalization. It is possible that PKC-mediated Thr-258 phosphorylation may have a modulatory role in NET regulation and in concert with Ser-259 phosphorylation dictates NET endocytosis. It has been shown that although Thr-189 phosphorylation of the Na-K-Cl cotransporter is necessary for activation of NKCC1, phosphorylation at Thr-184 and Thr-202 is only modulatory (56). In GAT1, PKC-dependent phosphorylation of serine residues can be revealed only when tyrosine phosphorylation is eliminated (41). As observed in the double mutant, a portion of PKC-induced phosphorylation still remained in the N’ + C’ + S502A + DM mutant even after elimination of all potential phospho-sites, including the PKC motif. These observations along with the fact that the double mutant exhibits altered kinetic properties suggest that when both Thr-258 and Ser-259 residues are mutated together, this double mutation or conformational change occurring because of mutation exposes other PKC-sensitive sites for phosphorylation. Mutations could distort transporter structure nonspecifically conferring altered phosphorylation/regulation. It is possible that other kinases or regulatory factors downstream of PKC activation may be involved. Regulatory mechanisms may also differ between cell types because of differential expression of PKC isoforms or other regulatory factors. Regardless of these speculations, our ability to explain all the reasons for this unusual behavior of the double mutant is limited by the current state of our understanding, and the present study does not rule out other mechanisms and/or phosphorylation of other sites involved in NET trafficking.

When controlled for equal protein expression levels, the double and single mutants exhibit elevated basal phosphorylation compared with that of WT-hNET. It is possible that other phosphosites not exposed previously might be available for phosphorylation to other kinases after mutation (of Ser-258 or Thr-259 or both). However, the motifs and signals involved in maintaining NET basal phosphorylation as well as the role of NET basal phosphorylation remain to be explored. Recently, we have demonstrated that constitutively active p38 MAPK is involved in maintaining SERT basal phosphorylation and constitutive expression (33). The basal phosphorylation of a protein kinase G/p38 MAPK-insensitive SERT G56A variant is elevated and cannot be further phosphorylated by PKG activation (32). It is possible that NET phosphorylation on other potential kinase-specific serine and threonine residues may be modulated by PKC-dependent phosphorylation of Thr-258 or Ser-259 or both in such a way that loss of PKC-induced NET regulation/endocytosis (as observed in the double mutant) may leave signaling pathways controlling basal phosphorylation unopposed.

The amino acid sequence at ICL2 region encompassing Thr-258 and Ser-259 is identical in DAT, NET, and SERT. However, recent elucidation of the crystal structure of leucine transporter (LeuTα), a bacterial homolog of neurotransmitter transporters, reveals very different amino acids at this region, and residues corresponding to Thr-258 and Ser-259 are located in TMD5 of LeuT (57) and would thus not be expected to be available for phosphorylation. It might be explained simply by structural differences between the NET and LeuT, i.e. LeuT shares only about 20–25% sequence identity with mammalian neurotransmitter transporters compared with a 64–71% sequence identity between DAT, NET, and SERT (58). Another and more likely explanation is that Thr-258 and Ser-259 only might be exposed in certain conformation during the transport cycle and/or to allow phosphorylation in the milieu of the lipid-mobile phase of mammalian plasma membrane; hence, the LeuT structure only represents a single snapshot of a highly dynamic protein in which both the putative extracellular and intracellular gates are closed. Interestingly, a recent study using cysteine-scanning mutagenesis showed that in SERT, the region between TMD4 and TMD5 encompassing Thr-276 and Ser-277 (Thr-258 and Ser-259 in NET) is accessible from the cytoplasmic side to cysteine-reactive reagents suggesting that ICL2 is much longer than the initially predicted sequence, at least in certain conformations (59).

The regulation of NET by neurokinin 1 receptor has several physiological implications. Locus coeruleus neurons are innervated by SP-containing fibers (60) and express NK1 receptors (61). Studies have shown a relationship between neurokinins and the noradrenergic system (9, 62–64). Excessive placental secretion of neurokinins during the third trimester has been linked to pre-eclampsia (65, 66). In addition to hypertensive effects manifested in pre-eclampsia, excess neurokinins might alter NE levels and hence NE signaling contributing to altered vascular function in the placenta. Thus, a close functional relationship might exist between neurokinin receptor activation and placental NE transport in the maintenance of a normal pregnancy.
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