Regulated Trafficking of the Human Dopamine Transporter

CLATHRIN-MEDIATED INTERNALIZATION AND LYSOSOMAL DEGRADATION IN RESPONSE TO PHORBOL ESTERS*

(Received for publication, July 22, 1999, and in revised form, September 8, 1999)

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The dopamine transporter (DAT) mediates the reuptake of dopamine into presynaptic neurons. This process of reaccumulation effectively reduces extracellular dopamine concentrations and limits activation of both presynaptic and postsynaptic dopamine receptors. The DAT is the primary target of psycho-stimulant drugs, which block the reuptake of released dopamine, resulting in an increase in synaptic dopamine levels (reviewed in Ref. 1). Functional loss of dopamine transport either through pharmacological inhibition (2) or genetic knockdown (3) results in profound physical, physiological, and behavioral changes. Given the essential role played by the DAT in the modulation of dopaminergic neurotransmission, the regulation of transporter activity is of considerable interest.

Cloning of the DAT and elucidation of its amino acid sequence revealed the presence of several consensus sites for protein kinase C (PKC) phosphorylation (3–5). Inhibition of DAT activity in response to activation of PKC by phorbol 12-myristate 13-acetate (PMA) has been observed in striatal synaptosomes and in several systems using cloned transporters expressed in either mammalian cells or Xenopus oocytes (6–11). Increased phosphorylation of the DAT in response to phorbol esters has been demonstrated in both endogenous and exogenous expression systems; however, no direct link between DAT phosphorylation and changes in transporter activity has been established (10, 11). The inhibition of DAT activity is the result of a decrease in V_{max}, with little or no change in the apparent affinity of the transporter for substrate, implying a decrease in the number of functional transporters at the surface of the cell. Activation of PKC also results in a decrease in the number of surface binding sites for nontransported inhibitors of the DAT (7–9). Furthermore, the inhibition of uptake activity observed in Xenopus oocytes expressing the human DAT (hDAT) is accompanied by a decrease in membrane capacitance, suggesting that the inhibition of uptake activity may be due to increased endocytosis of the transporter (9). Activation of PKC has also been suggested to result in changes in the subcellular localization of hDAT (12). However, high intracellular levels of DAT associated with transient overexpression systems made it difficult to evaluate the difference in transporter distribution by indirect immunofluorescence. Although internalization of the DAT as a mechanism of PMA-mediated inhibition remains a compelling possibility, this phenomenon has yet to be examined effectively. Likewise, changes in the trafficking of the transporter molecules in response to PKC activation and the cellular pathways involved have not been determined.

The use of green fluorescent protein (GFP) fusion proteins has provided the opportunity for real time optical analysis of protein trafficking in individual cells. To understand the cellular mechanisms underlying the PMA-mediated inhibition of DAT activity, we have generated a line of Madin-Darby canine kidney (MDCK) cells that stably express a GFP-tagged hDAT. This cell line has allowed us to directly visualize the subcellular localization of the DAT and observe the trafficking of the transporter molecules over time. We show that the activity of GFP-DAT is rapidly inhibited in the presence of phorbol esters and that this inhibition is the result of a significant loss of transporter protein from the plasma membrane. Internalization of hDAT into early endosomes is due to increased endocytosis through association with clathrin-coated pits (CCPs). Once internalized, the transporters transit through the endosomal/lysosomal pathway and are ultimately degraded.
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EXPERIMENTAL PROCEDURES

Materials—Cocaine and dopamine were purchased from Research Biochemicals International. Tunicamycin, aprotinin, antipain, chymostatin, leupeptin, pepstatin A, pargyline, anti-uvomorulin (E-cadherin) monoclonal antibody (DECMa-1), cycloheximide, nocodazole, and chloroquine were purchased from Sigma. Phenylmethylsulfonyl fluoride was obtained from Life Technologies, Inc. Forskolin, 3-isobutyl-1-methylxanthine (IBMX), PMA, 4-α-phorbol, staurosporine, and bafilomycin A1 were purchased from Calbiochem. Anti-actin antibody was from Roche Molecular Biochemicals, and lactacystin was obtained from Corixa Corp.

Plasmid Construction, Development of Stable Lines, and Cell Culture—The hDAT cDNA was inserted between the KpnI and XbaI sites of pEFGP-C1 (CLONTECH), creating the plasmid pEFGP-hDAT, which expresses a protein with enhanced green fluorescent protein directly fused to the amino terminus of the hDAT.

MDCK cells (ATCC) were grown to approximately 80% confluence in 35-mm tissue culture dishes and exposed to a solution containing 1.5 μg of plasmid DNA (pEFGP-hDAT or pEFGP-C1) and 40 μg Lipo-lectaMINE reagent (Life Technologies, Inc.) in serum-free Dulbecco’s modified Eagle’s medium for 5 h, after which the DNA/cationic lipid solution was replaced by Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (growth medium) containing 10 units/ml penicillin and 10 μg/ml streptomycin. 72 h post-transfection, the cells were plated at low density in growth medium containing 0.5 mg/ml G418 (Life Technologies, Inc.). Resistant colonies were selected and screened for expression of GFP-DAT or GFP by fluorescence microscopy. Cell lines expressing a moderate level of each protein were chosen for use in this study and are referred to as MDCK-GFP-DAT and MDCK-GFP, respectively.

Transport Assay—MDCK-GFP-DAT cells were grown to confluence in 12-well dishes, treated as indicated, and assayed for uptake activity essentially as described (13). Briefly, uptake was initiated by the addition of 100 nM [3H]dopamine (NEN Life Science Products) with or without cocaine. Uptake was allowed to continue for 3 min at room temperature. Background uptake was determined by assaying MDCK-GFP cells in parallel experiments. Specific uptake was considered to be the total uptake in MDCK-GFP-DAT cells minus the background uptake in MDCK-GFP cells after normalization for protein content.

Cell Surface Biotinylation and Western Blotting—Cell surface biotinylation was performed as described (14). MDCK-GFP-DAT cells were grown to near confluence in 6-well plates and incubated with biotinylation buffer (150 mM NaCl, 2 mM CaCl₂, 10 mM triethanolamine, pH 7.5) containing 2 mg/ml NHS-SB-ss-biotin (Pierce). The biotinylation reaction was quenched, and the cleared supernatants of cell extracts were incubated with UltraLink Immobilized NeutrAvidin (Pierce). The Neutravidin beads were washed and incubated for 30 min at room temperature. Background activity was determined by assaying MDCK-GFP cells in parallel experiments. Specific uptake was considered to be the total uptake in MDCK-GFP-DAT cells minus the background uptake in MDCK-GFP cells after normalization for protein content.

RESULTS

Characterization of MDCK Cells Stably Expressing GFP-DAT—The regulation of DAT activity by intracellular signaling mechanisms has been demonstrated by a number of studies. Results obtained using either biochemical and/or electrophysiological approaches have implied that the cellular trafficking of the carrier plays an essential role in the regulation process. However, changes in the distribution, trafficking, and fate of the DAT in response to second messenger activation have not been explored in detail. The use of a GFP-DAT fusion protein offers a means to visualize the movement of the carrier over time in live, stably transfected cells and has the potential to provide important insights into the mechanism of DAT regulation.

MDCK-GFP-DAT cells were examined for expression of GFP-DAT. Uptake of tritiated dopamine was robust in cells stably transfected with GFP-DAT (MDCK-GFP-DAT), whereas cells expressing GFP alone (MDCK-GFP) showed no accumulation of radiolabeled substrate (Fig. 1A). Uptake was linear over time for at least 15 min at room temperature (data not shown). Dopamine uptake in MDCK-GFP-DAT cells was inhib-
ined more than 90% by 100 μM cocaine (Fig. 1A), indicating that dopamine accumulation was specifically mediated by GFP-DAT.

Western blot analysis of cell extracts from MDCK-GFP-DAT, MDCK-GFP, or untransfected MDCK cells that were probed with polyclonal anti-GFP antisera showed an immunoreactive species centered at approximately 108 kDa (Fig. 1B) only in the GFP-DAT-expressing cells. This apparent molecular mass is approximately 27 kDa greater than that reported for wild-type hDAT (17), consistent with the presence of the GFP tag. Correspondingly, cells expressing only GFP exhibited a single immunoreactive species of approximately 27 kDa. A second band, centered at approximately 66 kDa, was observed in GFP-DAT-expressing cells but not in cells expressing GFP alone. To determine the identity of this band, MDCK-GFP-DAT cells were incubated for 16 h in the presence of tunicamycin to block N-linked glycosylation. Immunoblots of lysates from tunicamycin-treated cells showed significantly reduced levels of the 108-kDa band (Fig. 1C). The lower band appeared to migrate at 58 kDa, somewhat more rapidly than that seen in untreated MDCK-GFP-DAT cells. This band is the same size as that found in lysates treated with peptide N-glycosidase F, suggesting that it represents the unglycosylated form of the DAT. Furthermore, cells solubilized in lysis buffer containing a mixture of protease inhibitors exhibit the same immunoreactive species as those solubilized in lysis buffer alone (Fig. 1C). Taken together, these data suggest that the lower molecular mass species represents immature, core-glycosylated protein rather than a proteolytic degradation product.

Activation of PKC by phorbol esters has been shown to inhibit the activity of the hDAT in a number of cell types (6–11). We assayed MDCK-GFP-DAT cells for dopamine uptake after treatment with activators and inhibitors of cell signaling pathways. When treated with PMA, MDCK-GFP-DAT cells showed a reduction in uptake activity of approximately 70% (Fig. 2A). In contrast, exposure of MDCK-GFP-DAT cells to forskolin or IBMX, potent activators of the cyclic AMP-dependent protein kinase pathway, had no effect on the accumulation of radiolabeled substrate as compared with untreated control cells or cells treated with vehicle alone. An inactive isomer of PMA, 4-α-phorbol, had no significant effect on dopamine uptake, suggesting that transport inhibition was specific to PKC activation. Similarly, preincubation with staurosporine, a PKC inhibitor, effectively blocked the PMA-mediated inhibition of uptake activity, whereas staurosporine alone had no effect. In addition, when endogenous PKC activity was depleted by exposing the cells to 100 nM PMA for 16 h, the subsequent addition of new PMA no longer produced inhibition of uptake activity (data not shown).

Dopamine uptake was also examined after incubation with PMA at concentrations ranging from 10 pM to 10 μM. Half maximal inhibition (IC50) occurred at a concentration of 3.2 ± 0.5 nM, and the inhibition saturated at concentrations over 30 nM (Fig. 2B). Inhibition of uptake by 100 nM PMA was maximal within 20 min, with a calculated T1/2 of 8.0 ± 0.8 min (Fig. 2B). Based on these determinations, a 20-min incubation with 100 nM PMA was used for all subsequent experiments unless otherwise indicated.

In either the presence or absence of PMA, dopamine uptake is both concentration-dependent and saturable (Fig. 2C). Kinetic analysis indicated that PMA reduced the maximal velocity of dopamine transport by approximately 66% compared with control cells exposed to vehicle alone (Vmax = 110 ± 6.5 versus 296 ± 10 pmol/min/mg of protein). However, the apparent affinity of the transporter for dopamine remained unchanged, with a Kp of 3.8 ± 0.7 μM in the absence of PMA and 3.8 ± 1.0 μM in the presence of PMA.

The characteristics of PMA-mediated inhibition of GFP-DAT activity in this experimental system are very similar to those observed with wild-type DAT, suggesting that the presence of the GFP moiety did not effect transporter function. The apparent affinity of the chimeric protein for dopamine was identical to that observed for wild-type rat DAT (Kp = 3.9 ± 0.9 μM) stably expressed in MDCK cells (data not shown). The affinity
MDCK-GFP-DAT cells were treated for 20 min at 37 °C with Me 2SO antibody. Incubation with PMA or vehicle control, fixed, and probed with DECMA-1 cadherin in MDCK-GFP-DAT cells. MDCK-GFP-DAT cells were incubated with PMA or vehicle control after a 15-min preincubation with staurosporine. MKK-GFP-DAT cells were incubated in the presence of 50 μM pargyline and 0.1 mM ascorbic acid with or without 10 μM dopamine or 100 μM cocaine for 20 min before treatment with PMA or vehicle control. 

Indirect immunofluorescence of E-cadherin in MKK-GFP-DAT cells. MKK-GFP-DAT cells were incubated with PMA or vehicle control, fixed, and probed with DECMA-1 antibody.

Of the transporter for substrate was also comparable with that determined for the wild-type hDAT in other model systems (7, 9). In the presence of PMA we observed a maximal inhibition of 60–70%. Although the extent of inhibition seen in previous studies is similar, it appears to vary depending on the model system used (7, 9, 12).

**Subcellular Distribution of GFP-DAT**—A decrease in V<sub>max</sub> without an accompanying decrease in affinity for substrate could arise from events that alter the turnover rate of the carrier or from a loss of functional transporter molecules at the cell surface. To determine whether a reduction in the number of GFP-DAT molecules at the plasma membrane underlies the PMA-mediated decrease in V<sub>max</sub> the cellular distribution of GFP-DAT was examined by confocal microscopy (Fig. 3). When PKC activity was stimulated by PMA, the majority of GFP-DAT fluorescence was punctate and intracellular with only minor levels of fluorescence seen at the plasma membrane. This is in stark contrast to untreated control MKK-GFP-DAT cells, where the transporter was found almost exclusively at the cell surface (Fig. 3A). A small amount of intracellular fluorescence was also observed in the control cells, most likely due to the presence of immature transporter protein transiting through the secretory pathway. The distribution of the fluorescent signal seen in cells treated with either vehicle alone or with activators of the PKA signaling pathway was identical to that seen in untreated control cells. The translocation of GFP-DAT in response to PMA is specific to PKC activation, as redistribution of the transporter was not seen in cells treated with 4-α-phorbol. Furthermore, PMA-mediated internalization of the carrier was effectively blocked by pretreatment with the PKC inhibitor staurosporine, whereas staurosporine alone had no effect on transporter localization. These observations are consistent with results seen for uptake activity under the same conditions (Fig. 2A).

In a recent report, the γ-aminobutyric acid (GABA) transporter GAT1, was shown to undergo changes in cellular distribution after incubation with GABA or with SKF89976A, a specific inhibitor of GAT1 (18). To determine whether the presence of either substrate or inhibitor had an effect on GFP-DAT localization, MKK-GFP-DAT cells were stimulated with PMA after incubation with either dopamine or cocaine. Even though the duration of exposure to substrate or inhibitor was greater than that necessary to produce complete redistribution of GAT1, there was no change in the cellular localization of GFP-DAT (Fig. 3B). Furthermore, the presence of either dopamine or cocaine had no effect on the PMA-mediated internalization of the transporter.

We also examined the effect of PKC activation on the localization of another cell surface protein, E-cadherin. Even though E-cadherin is rapidly endocytosed in MDCK cells when internal stores of ATP are depleted (19), incubation of MKK-GFP-DAT cells with PMA had no effect on E-cadherin localization under conditions that induce complete redistribution of GFP-DAT in the same cells (Fig. 3C). This result suggests that internalization of GFP-DAT in response to PMA does not reflect a general increase in vesicular traffic from the cell surface but instead involves a more selective increase in the endocytosis of GFP-DAT.

The appearance of transporter protein in intracellular vesicular compartments after activation of PKC could result from either internalization of DAT from the plasma membrane or inhibition of trafficking of the carrier to the cell surface. To distinguish between these possibilities, protein synthesis was inhibited to deplete intracellular stores of GFP-DAT. When MKK-GFP-DAT cells were incubated with cycloheximide for 4 h, intracellular fluorescence was no longer observed, and Western blot analysis showed a complete loss of the 66-kDa core glycosylated form of the carrier (data not shown). This result suggests that under these conditions all of the transporter in the cell is fully glycosylated and is found predominantly or exclusively at the plasma membrane. Cell surface biotinylation of cycloheximide-treated cells exposed to PMA for 5–60 min showed a progressive loss of GFP-DAT protein from the plasma membrane (Fig. 4A). The amount of surface GFP-DAT in control cells remained unchanged over the same time period. Quantification of the biotinylated GFP-DAT by densitometry shows a gradual decrease in signal intensity over time with a loss of approximately 90% within 1 h (Fig. 4B). However, no significant change was seen in cells treated with vehicle alone. Because previous results suggested that no more than 70% uptake activity was lost in cells treated for 60 min with PMA in the absence of cycloheximide (refer to Fig. 2), we compared uptake activity in control and cycloheximide-treated cells. For the first 10 min of PMA exposure, inhibition of uptake activity was equivalent in untreated and cycloheximide-treated cells (Fig. 4C). However, after 15 min the magnitude of inhibition of uptake activity was greater in cycloheximide-treated cells than in untreated cells. This trend continued through a 60-min exposure to PMA. In addition, inhibition was maximal at approximately 70% within 20 min in untreated cells, whereas uptake activity continued to decrease in cycloheximide-treated cells, reaching a maximal inhibition of nearly 90% at 45 min (Fig. 4C).

These results suggested that less GFP-DAT remained at the cell surface after PKC activation when protein synthesis was inhibited. To determine whether inhibition of protein synthesis altered the localization of GFP-DAT after PMA treatment, we examined the PMA-mediated redistribution of GFP-DAT in live cells over time in the presence or absence of cycloheximide. MKK-GFP-DAT cells grown on glass coverslips were incubated with cycloheximide or vehicle alone and transferred to a heated chamber system. PMA was added, and confocal images were collected every 5 min over a 60-min time period. At the time of PMA application, GFP fluorescence was seen primarily at
Values are the percentage of uptake compared with Me 2SO-treated controls. Focal images were collected over a period of 60 min after treatment with PMA and without cycloheximide (C). MDCK-GFP-DAT cells were incubated for 4 h with cycloheximide before being transferred to the chamber system. Under these conditions, all of the fluorescent signal was seen at the cell surface before the addition of PMA. When cycloheximide-treated cells were exposed to PMA, we again observed an increase in intracellular fluorescence with a concomitant decrease in cell surface fluorescence. However, in contrast to cells not preincubated with cycloheximide, the fluorescent signal at the plasma membrane completely disappeared.

**Transit of GFP-DAT through the Endosomal/Lysosomal Pathway**—Having established that the population of DAT molecules found at the plasma membrane were internalized in response to PMA, we explored the cellular mechanism by which this endocytic event occurs. The GTPase dynamin associates with CCPs and plays an essential role in the budding of endocytic vesicles (20, 21). We utilized a dominant negative mutant of dynamin 1 to effectively block clathrin-mediated endocytosis. MDCK-GFP-DAT cells were infected with a vaccinia virus expressing either wild-type dynamin 1 or K44E, a dominant negative mutant of dynamin 1 (22). Eight h after infection, the cells were treated with PMA or vehicle control in the presence of labeled transferrin. Cells infected with vaccinia virus expressing the dominant negative mutant of dynamin exhibited no accumulation of transferrin, as expected based on the results of previous studies (22, 23). Dynamin K44E also blocked the PMA-mediated internalization of GFP-DAT, indicating that this process requires functional budding of CCPs (Fig. 5A). In contrast, when cells were infected with a vaccinia virus expressing wild-type dynamin 1 (Fig. 5A) or with wild-type vaccinia virus (data not shown), PMA still induced the translocation of GFP-DAT, and transferrin was accumulated normally into endosomes.
compartment analysis was used to determine the identity of vesicles into which the transporter was internalized. Early endosomes were labeled by incubating the MDCK-GFP-DAT cells with fluorescently labeled canine transferrin. After 20 min of exposure to both PMA and labeled transferrin, GFP-DAT fluorescence only partially overlapped with that of transferrin (Fig. 5B). However, if the cells were preincubated with bafilomycin A1 and nocodazole, conditions that have been shown to block maturation of early endosomes (24), nearly all of the GFP fluorescence was seen in vesicles containing labeled transferrin. One explanation for this finding is that GFP-DAT and transferrin are initially taken up into the same vesicle population but later diverge. To prove this, we examined the colocalization of GFP-DAT and transferrin at an earlier time point after the application of PMA. To obtain a detectable signal within a shorter time period, MDCK-GFP-DAT cells were preincubated for 1 h with labeled transferrin at 4 °C, followed by treatment with PMA for 5 min at 37 °C. Under these conditions, essentially all of the GFP fluorescence was found in vesicles that also contained transferrin.

We also examined the colocalization of GFP-DAT and an endogenous MDCK lysosomal membrane glycoprotein recognized by the monoclonal antibody AC17 (25). After a 20-min incubation with PMA, only a small fraction of the GFP-DAT colocalized with the lysosomal marker (Fig. 5C). Even after 60 min of PMA treatment, the GFP signal only partially overlapped with AC17 labeling. The failure to see significant colocalization of GFP-DAT and the AC17 antigen may have been due to rapid degradation of the GFP label or to quenching of the fluorescent signal that occurs at low pH (26). Therefore, lysosomal acidification and the activity of lysosomal proteases were blocked by incubating the cells with NH₄Cl before and during PMA treatment. Under these conditions, virtually all of the GFP fluorescence was found in AC17-labeled vesicles, which exhibit a dramatic increase in size. Similar results were obtained when chloroquine was used to inhibit lysosomal degradation (data not shown).

Degradation of Transporter Protein after PKC Activation—The presence of GFP-DAT in lysosomes suggested that the carrier was being targeted for degradation. Therefore, we used Western blot analysis to determine the amount of GFP-DAT protein in the cells over time after incubation with PMA. MDCK-GFP-DAT cells were incubated in the presence of cycloheximide for 4 h to block protein synthesis. Under these conditions, trichloroacetic acid precipitation of radiolabeled proteins showed that greater than 97% of protein synthesis was inhibited, and protein synthesis did not recover during the time course of the experiment (data not shown). Cycloheximide-treated cells were preincubated with inhibitors of either lysosomal or proteasomal degradation and then exposed to PMA. After 20 min, excess PMA was washed away, and incubation was continued in the presence of cycloheximide and proteinase inhibitors. The cells were harvested at various time points after removal of PMA from the culture medium. One h after PMA treatment, there was a distinct reduction in GFP-DAT protein levels (Fig. 6). After 2 h, GFP-DAT protein was barely visible in PMA-treated cells, and by 3 h it was completely absent. GFP-DAT levels remained unchanged, however, in cells incubated with the lysosomotropic amines NH₄Cl or chloroquine, either with or without PMA stimulation. In contrast, lactacystin or the vinyl sulfone ZL3VS, specific inhibitors of proteasomal degradation, failed to block PMA-mediated degradation of GFP-DAT. When protein synthesis was not inhibited by cycloheximide, GFP-DAT levels were significantly reduced after incubation with PMA, but the protein was never entirely lost, even 8 h after exposure to PMA (data not shown). The mature GFP-DAT protein remaining after 3 h under these conditions was assumed to be newly synthesized protein. Taken together, these data indicate that PKC activation results in the complete lysosomal degradation of mature GFP-DAT molecules.

**DISCUSSION**

Several members of the Na⁺Cl⁻-dependent neurotransmitter transporter family, including the dopamine, serotonin, norepinephrine, taurine, and GABA transporters, have been shown to be acutely regulated upon activation of PKC by phorbol esters (8, 27–30). A number of studies have implicated internalization of the transporters as a possible means of modulating transporter activity (7, 9, 12, 31, 32). Cell surface biotinylation of the serotonin and norepinephrine transporters provided the first direct evidence that the surface expression of these carriers was reduced after treatment with PMA (31, 32). Recent reports suggest that the dopamine and norepinephrine transporters are redistributed after PKC activation when examined by indirect immunofluorescence (12, 31). However, the results of these studies were obscured by high intracellular levels of transporter, making interpretation of the data difficult. The work presented here provides clear evidence that regulation of DAT activity through PKC activation is the result of rapid internalization of the carrier. Furthermore, we show that PMA-mediated internalization of the DAT is due to an increase in endocytosis through CCPs and that, following internalization, the transporter is targeted to the lysosomal pathway, where it is ultimately degraded. Under normal conditions, the majority of DAT protein is found at the cell surface (Fig. 3). Only the fully glycosylated carrier is found at the plasma membrane, as demonstrated by the complete absence of the unglycosylated species on blots of surface-labeled proteins (Fig. 4A). In response to stimulation by PMA, a PKC-mediated phosphorylation event leads to the rapid internalization of the transporter (Fig. 3). Whether PMA-mediated internalization is the result of the direct phosphorylation...
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Internalization of the hDAT remains unclear. The primary amino acid sequence of the hDAT contains multiple consensus sites for PKC phosphorylation, and increased phosphorylation of the hDAT has been shown in response to PKC stimulation (10, 11, 33). However, mutation of the canonical PKC phosphorylation sites within the GABA and glycine transporters, which are also regulated by PKC activation, has no effect on PKC-mediated inhibition of transporter activity (30, 34). Therefore, internalization of these transporters as well as of the hDAT may be the result of phosphorylation of an unidentified protein that is associated with the carriers at the cell surface.

In the MDCK cell system, essentially all of the transporter found at the cell surface is internalized in response to PKC activation. In the presence of cycloheximide, when all of the detectable transporter molecules are localized to the plasma membrane, there is a nearly complete loss of both uptake activity (Fig. 4c) and surface fluorescence (Fig. 4b) in response to application of PMA. There is less inhibition of uptake activity when protein synthesis is allowed to continue (Fig. 4c), suggesting that under normal conditions the plasma membrane is repopulated by intracellular stores of DAT. These findings are consistent with the idea that internalization of the DAT requires the phosphorylation of a protein that is associated with the DAT at the cell surface. However, the possibility remains that translocation is the result of direct phosphorylation of the DAT if PKC preferentially phosphorylates the mature transporter molecules found at the cell surface. The observation that PMA-mediated translocation of the DAT is independent of the presence of substrate or inhibitor (Fig. 3b) suggests that conformational changes resulting from the binding of dopamine or cocaine are not important to either phosphorylation or translocation.

The DAT is internalized through a clathrin-mediated mechanism after activation of PKC. We were able to completely block PMA-mediated translocation of DAT with a dominant negative mutant of dynamin 1 (Fig. 5a). Receptor-mediated endocytosis of transferrin, a clathrin-dependent process, was also completely abolished under these conditions. Dynamin has also been suggested to play a role in the budding of caveolae (35, 36). However, internalization of the DAT due to an increase in endocytosis through this pathway is unlikely, as PMA inhibits the formation of caveolae in kidney epithelial cells (37). Furthermore, digitonin, filipin, and nystatin, inhibitors of caveolae-mediated endocytosis, had no effect on DAT translocation, even at concentrations in excess of those demonstrated to effectively block the formation of caveolae (38) (data not shown). In contrast, internalization of the DAT was almost completely abolished by either chlorpromazine or monodansylcadaverine, potent inhibitors of clathrin-mediated endocytosis (data not shown).

Our observation that the DAT is degraded after PMA-mediated internalization implies that this process is irreversible. Others have suggested that translocation of the hDAT in response to PMA is bidirectional (12), but this was examined only in baculovirus-infected SF9 cells, which may vary significantly from mammalian cells in protein trafficking. Furthermore, as protein synthesis was not inhibited, the possibility that the reappearance of the transporter at the cell surface simply reflects repopulation by intracellular stores cannot be eliminated. We did not observe any recovery of uptake activity or alteration in subcellular localization in MDCK-GFP-DAT cells under the same conditions (data not shown). We cannot exclude the possibility that the DAT returns to the cell surface in response to the appropriate stimulation; however, the nature of this signal remains unknown.

PMA is known to modulate the surface expression of a number of biologically important proteins, including the epidermal growth factor receptor (39), the Na+/glucose cotransporter (40), chemokine receptors (41, 42), and the surface glycoprotein CD4 (43). A normal constituent of lymphoid cells, CD4 is rapidly internalized in response to activation of PKC by either PMA or by stimulation of the T cell receptor (44). In a manner similar to the DAT, CD4 internalization is mediated by an increase in clathrin-associated endocytosis and is followed by lysosomal degradation of the protein (45, 46). The signals that direct phosphorylated CD4 to CCPs and to the lysosome are found within the cytoplasmic tail of the molecule. The two most common signals for targeting of membrane proteins to coated pits are tyrosine- and dileucine-based motifs (reviewed in Ref. 47). The carboxyl-terminal tail of the hDAT contains two tyrosine residues. One of these tyrosine residues, Tyr-578, is positioned too close to a membrane-spanning domain to serve as a signal for accumulation in coated pits (48). Although the importance of the second tyrosine (Tyr-593) to internalization of the transporter has yet to be determined, it is not flanked by any of the amino acid residues normally associated with canonical endocytosis signals (47). Although the carboxyl tail of the hDAT does not contain an obvious dileucine motif, there is a dileucine sequence (Leu-440–Leu-441) within the putative intracellular loop between transmembrane domains 8 and 9. Whether or not this dileucine sequence is involved in PMA-mediated internalization of the DAT has not been established.

The idea that internalization of the DAT is a consequence of its association with another protein after PKC activation remains an intriguing one. The regulation of CD4 trafficking during viral infection provides an interesting illustration of such a mechanism. Like PKC activation, infection of T cells by human immunodeficiency virus induces the internalization and degradation of CD4 (49). This is attributed to the presence of the virally encoded protein Nef (50), which associates directly with CD4 (51), and directs both the accumulation of CD4 first in CCPs (52) and then in lysosomes (53). Desensitization of the β2-adrenergic receptor also involves internalization through its interaction with a secondary protein. In the presence of agonist, phosphorylated β2-adrenergic receptor binds to the connector protein β-arrestin, which promotes the accumulation of β2-adrenergic receptor in CCPs through a direct interaction with clathrin cages (54).

Redistribution of membrane proteins is proposed to play an important role in synaptic plasticity. Stimulation of long term depression has recently been demonstrated to induce the internalization of the AMPA-type glutamate receptor in hippocampal cultures (55). The kinase-dependent regulation of other molecules important to synaptic function has also been shown to be due to increased endocytosis. The intracellular accumulation of both muscarinic acetylcholine receptors and GABA type A receptors is seen after activation of PKC by phorbol esters (56, 57). Although the fate of the AMPA and GABA type A receptors after internalization remains unknown, the muscarinic receptor is subsequently degraded. Such changes in trafficking of receptors, ion channels, and transporters could serve as an important mechanism for regulating neurotransmitter signaling and synaptic strength.

Acknowledgments—Monoclonal antibody AC17 was a kind gift from Dr. Enrique Rodriguez-Boulan (Cornell, New York), and ZL-VS was supplied by Dr. Matthew Bogyo (Harvard Medical School). All vaccinia viruses were graciously provided by Dr. Gary Thomas (Vallum Institute).

REFERENCES
1. Amara, S. G., and Sanders, M. S. (1998) Drug Alcohol Depend. 51, 87–96
2. Ritz, M. C., Lamb, R. J., Goldberg, S. R., and Kuhar, M. J. (1987) Science 237, 1219–1223
