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The Dopamine Transporter Constitutively Internalizes and Recycles in a Protein Kinase C-regulated Manner in Stably Transfected PC12 Cell Lines*

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The dopamine transporter (DAT) removes dopamine from the extracellular milieu and is potently inhibited by number of psychoactive drugs, including cocaine, amphetamines, and methylphenidate (Ritalin). Multiple lines of evidence demonstrate that protein kinase C (PKC) down-regulates dopamine transport, primarily by redistributing DAT from the plasma membrane to endosomal compartments, although the mechanisms facilitating transporter sequestration are not defined. Here, we demonstrate that DAT constitutively internalizes and recycles in rat pheochromocytoma (PC12) cells. Temperature blockades demonstrated basal internalization and reliance on recycling to maintain DAT cell surface levels. In contrast, recycling blockade with bafilomycin A_1 significantly decreased transferrin receptor (TfR) surface expression but had no effect on DAT surface levels, suggesting that DAT and TfR traffic via distinct endosomal mechanisms. Kinetic analyses reveal robust constitutive DAT cycling to and from the plasma membrane, independent of transporter expression levels. In contrast, phorbol ester-mediated PKC activation accelerated DAT endocytosis and attenuated transporter recycling in a manner sensitive to DAT expression levels. These data demonstrate constitutive DAT trafficking and that PKC-mediated DAT sequestration is achieved by a combination of accelerated internalization and reduced recycling. Additionally, the differential sensitivity to expression level exhibited by constitutive and regulated DAT trafficking suggests that these two processes are mediated by independent cellular mechanisms.

Dopaminergic neurotransmission is fundamental to a variety of central nervous system functions, including motor control (1, 2) and cognition (3). Aberrant DA[1] neurotransmission is implicated in Parkinson's disease (4, 5) and schizophrenia (6, 7), the symptoms of which are ameliorated by increasing and decreasing DA signaling, respectively. Once released into the synapse, the primary mechanism limiting extracellular DA concentrations is presynaptic re-uptake mediated by the plasma membrane DAT. DAT belongs to the Na^+/Cl^--dependent transporter gene family (8, 9) and is potently inhibited by the addictive psychostimulants cocaine and amphetamine (10), making DAT a major psychostimulant target in the brain. Indeed, cocaine and amphetamines neither raise extracellular DA levels (11) nor produce hyperlocomotion (12, 13) in $DAT^{-/-}$ mice. A recent report also implicates DAT in nonvesicular DA release in the substantia nigra somatodendritic region (14). Hence the number of functional DATs present on the plasma membrane directly impacts dopaminergic signaling and psychostimulant efficacy.

Although once considered static resident plasma membrane proteins, a growing body of evidence demonstrates that DAT surface expression is highly dynamic. The best documented example is through acute PKC activation, which down-regulates DAT and its homologues by decreasing their plasma membrane presentation (8, 15, 16). Moreover, DAT surface presentation is acutely sensitive to the psychostimulants amphetamine (17) and cocaine (18, 19), which decrease and increase DAT surface levels, respectively. Taken together, these findings suggest that membrane trafficking is a fundamental mechanism governing DAT homeostasis and regulation. This hypothesis is further supported by evidence that the dominant negative dynamin mutant K44A blocks both PKC (20)- and amphetamine-mediated (17) DAT sequestration, suggesting that clathrin-mediated endocytosis is required for many transporter regulatory processes.

Our previous report (21) demonstrated that DAT basally distributes evenly between the plasma membrane and endosomal compartments, and that surface DAT translocates to the recycling endosome in response to PKC activation. A lingering question arising from these studies is whether PKC-induced DAT losses from the plasma membrane occur by accelerating DAT internalization, attenuating DAT recycling, or a combination of both. Further, it is unknown whether DAT significantly traffics under basal conditions. Here, we tested the hypotheses that DAT constitutively cycles to and from the plasma membrane and that PKC activation modulates already existent DAT trafficking. Our results demonstrate surprisingly robust constitutive DAT trafficking, which is modulated in response to

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[1] The abbreviations used are: DA, dopamine; DAT, dopamine transporter, PKC, protein kinase C; TfR, transferrin receptor; PMA, phorbol 12-myristate 13-acetate; NET, norepinephrine transporter; SERT, serotonin transporter; GAT1, γ-aminobutyric acid transporter 1; PBS, phosphate-buffered saline; MesNa, mercaptoethanesulfonic acid; NHS-SS, sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate; ANOVA, analysis of variance.

PKC activation, suggesting that DAT surface expression is highly dynamic even under basal conditions.

EXPERIMENTAL PROCEDURES

Materials—Bafilomycin A_1 and GBR12909 were from Tocris-Cookson (Ellisville, MO). Rat monoclonal DAT antibody and all horseradish peroxidase-conjugated secondary antibodies were from Chemicon (Temecula, CA). Mouse anti-TfR antibody was from Zymed Laboratories Inc. (South San Francisco, CA). Rabbit anti-rab5A antibody and mouse anti-rab11 and anti-EEA1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were of the high possible quality and were obtained from Sigma unless otherwise noted.

Cell Culture and Uptake Assays—PC12 cells stably expressing the human DAT (DAT-PC12) were cultured at 37 °C, 10% CO_2 as described previously (21). The cell line 4.27.37 was used for the majority of experiments, and cell lines 5.11.18 and 5.11.33 were also used where specified. For uptake, cells were plated in triplicate on poly-D-lysine-coated 24-well plates 1 day prior to performing the assays. Cells were rinsed and preincubated in KRH buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.18% glucose, 10 mM HEPES, pH 7.4) at either 18 or 37 °C for 30 min in the presence of 100 nM desipramine to block endogenous NET activity. Cells were shifted to 18 °C for 2 min, and uptake was initiated with 1.0 μM [^{3}H]DA (3,4-[*ring*-2,5,6,-^{3}H]DA, PerkinElmer Life Sciences) containing 10^{-5} M pargyline and 10^{-5} M ascorbic acid. Assays proceeded for 10 min (18 °C) and were terminated by rapidly washing cells with ice-cold KRH buffer. Cells were solubilized in 1% SDS, and accumulated radioactivity was determined by liquid scintillation counting. Nonspecific uptake was defined in the presence of 100 nM desipramine and 10 μM GBR12909. Data analysis was performed using Microsoft Excel and KaleidaGraph (Synergy Software). Statistical analysis was performed using Instat (GraphPad Software).

Cell Surface Biotinylation—DAT-PC12 cells, grown to ~75% confluency on poly-D-lysine-coated six-well plates, for the designated times and temperatures were washed and incubated in PBS^{2+}/B/g (PBS supplemented with 1.0 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2% bovine serum albumin, 0.18% glucose). Plasma membrane proteins were biotinylated with sulfo-NHS-SS-biotin essentially as described previously (21). Cells were lysed in radioimmune precipitation buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1.0 mM EDTA, 0.1% SDS, 1.0% Triton X-100, 1.0% sodium deoxycholate) containing protease inhibitors (1.0 mM phenylmethylsulfonyl fluoride, and 1.0 μg/ml each leupeptin, aprotinin, and pepstatin), and cellular debris was removed by centrifugation. Biotinylated and nonbiotinylated proteins from equivalent amounts of total cellular protein were separated by streptavidin pull-down, and the resulting eluents and supernatants were assessed by immunoblot as described previously (21). Nonsaturating, immunoreactive bands were quantitated either by scanning densitometry or with a CCD camera gel documentation system (ChemiDoc, Bio-Rad), and nonsaturating bands were quantitated with Quantity One software (Bio-Rad).

Internalization Assay—DAT-PC12 cells were plated on duplicate poly-D-lysine-coated six-well plates and underwent biotinylation with 2.5 mg/ml sulfo-NHS-SS-biotin as described. Following biotinylation, one set of cells was washed with PBS^{2+} (3 × 2 ml) and kept at 4 °C to determine the total initial surface DAT and strip efficiencies. To initiate endocytosis, cells in the duplicate plate were washed repeatedly with prewarmed (37 °C) PBS^{2+}/B/g containing either 1 μM PMA or vehicle and incubated with the same solutions for 10 min at 37 °C. To assure rapid temperature shift to 37 °C, temperatures were monitored in representative wells and were found to be completely shifted to 37 °C in less than 2 min. Endocytosis was rapidly stopped by placing plates immediately in a slushy ice bath and washing 3 × 2 ml with ice-cold PBS^{2+}. Residual cell surface biotin was stripped by incubating cells twice for 25 min with freshly prepared 50 mM MesNa in NT buffer (150 mM NaCl, 1 mM EDTA, 0.2% bovine serum albumin, 20 mM Tris, pH 8.6). Strip efficiency was determined for each experiment on biotinylated cells kept in parallel at 4 °C and averaged >95%. Cells were lysed in radioimmune precipitation buffer with protease inhibitors, biotinylated proteins were separated from nonbiotinylated proteins by streptavidin pull-down from equivalent amounts of cellular protein, and samples were analyzed by SDS-PAGE and immunoblot as described above. Immunoreactive bands were detected with a cooled, CCD camera (ChemiDoc system, Bio-Rad), and bands in the linear range of detection were quantitated using Quantity One software (Bio-Rad). Relative DAT endocytic rates were calculated as the percent DAT internalized compared with total initial DAT surface pool.

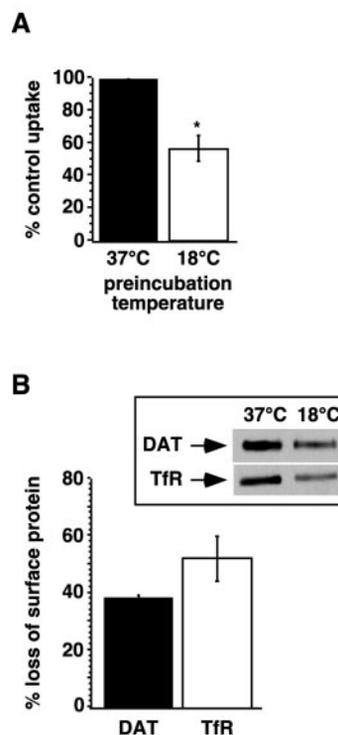*Biotinylation of Recycling DAT Pool*—DAT-PC12 cells plated on poly-

FIG. 1. **18 °C recycling blockade decreases DA transport and DAT surface expression in DAT-PC12 cells.** *A*, DA uptake assay of DAT-PC12 cells preincubated at 37 °C (*filled bar*) or 18 °C (*open bar*) for 30 min. Data are expressed as % control uptake ± S.E. *, $p < 0.005$ (Student's *t* test, $n = 4$). *B*, steady state cell surface biotinylation of DAT-PC12 cells. *Inset*, representative immunoblot showing surface DAT and TfR after incubation at 37 or 18 °C. *Bar graph*, averaged data expressed as % loss of 37 °C cell surface pool ± S.E. ($n = 3$, DAT; $n = 2$, TfR).

D-lysine-coated six-well plates were washed and preincubated in prewarmed PBS^{2+} for 20 min at 37 °C. Cells were incubated either three times for 15 min (for 45-min biotinylation) or three times for 10 min (for 30-min biotinylation) with 1.0 mg/ml sulfo-NHS-SS-biotin in PBS^{2+} at 37 °C. Following glycine quench, cells were lysed, and biotinylated proteins were separated from nonbiotinylated proteins by streptavidin pull-down and analyzed by SDS-PAGE and immunoblot as described above.

RESULTS

In our previous study of DAT-transfected PC12 cells (21), under steady state conditions we observed a sizeable intracellular DAT pool that localized to recycling endosomes, suggesting that DAT may be subject to considerable basal trafficking. In the present study, we tested the hypothesis that DAT constitutively traffics from and back to the plasma membrane and that DAT redistribution in response to PKC activation results from altering existing DAT trafficking kinetics.

Constitutive DAT Trafficking—To determine whether DAT undergoes constitutive membrane trafficking, we globally blocked endocytic recycling and assessed the resultant effects on DAT function and surface expression. First, we subjected DAT-PC12 cells to an 18 °C blockade, which is temperature-permissive to endocytosis but significantly attenuates exit from the early endosome (22–25). Thus, if DAT constitutively internalizes and recycles, we would expect DAT surface presentation to decrease during 18 °C incubations. A 30-min incubation at 18 °C reduced DA uptake by 52.7 ± 10.6% when compared with cells preincubated in parallel at 37 °C (Fig. 1*A*). Cell surface biotinylation revealed that the 18 °C treatment decreased DAT surface expression by 38.8 ± 6.1% as compared with control cells incubated in parallel at 37 °C (Fig. 1*B*), with no losses in total DAT (data not shown). DAT surface losses
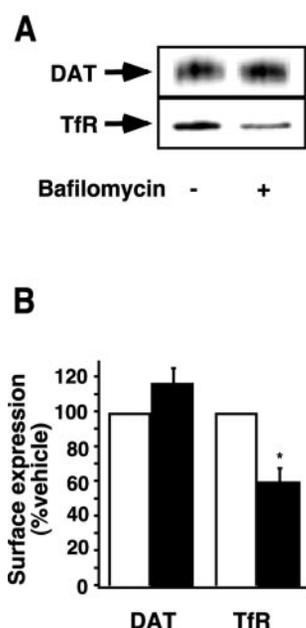
FIG. 2. **Bafilomycin reduces the surface expression of TfR but not DAT in DAT-PC12 cells.** *A*, representative immunoblot of cell surface DAT and TfR following 30-min treatment with vehicle or 0.5 μM bafilomycin A$_1$. *B*, quantitation of DAT and TfR surface levels following treatment with vehicle (*open bars*) *versus* 0.5 μM bafilomycin A$_1$ (*filled bars*). Data are expressed as % vehicle surface pool $\pm$ S.E. *, $p < 0.005$ compared with vehicle-treated cells (Student's *t* test, $n = 4$).

were not significantly different from those observed for TfR (Fig. 1*B*, 52.6 $\pm$ 7.9% of control), a protein that undergoes robust constitutive cycling.

DAT surface losses following the 18 °C incubation suggest that DAT constitutively internalizes under steady state conditions and that recycling is required to maintain steady state DAT surface levels. However, because 18 °C also attenuates exit from the trans-Golgi network, loss of surface DAT at 18 °C may simply reflect normal DAT turnover in the absence of biosynthetic replenishment. To rule out the contribution of newly synthesized DAT to the cell surface pool, we examined total and surface DAT while protein synthesis was blocked with cycloheximide. Cycloheximide treatment (10 μM) inhibited [^{35}S]methionine/cysteine incorporation into total cellular protein by >90% over a time span of 3 h but had no significant effect on either DAT surface levels or total DAT levels (ANOVA, $p = 0.28$, $n = 2$; data not shown). These results demonstrate that the biosynthetic contribution of DAT to the cell surface pool is not significant in the time frames in which our experiments were performed.

To further examine the role of recycling in maintaining DAT surface levels, we blocked constitutive recycling with the vacuolar ATPase inhibitor bafilomycin A$_1$, which blocks TfR recycling and reduces TfR surface expression (26). Bafilomycin treatment (0.5 μM) for 30 min reduced TfR plasma membrane expression to 60.6 $\pm$ 6.6% of that observed for vehicle-treated cells (Fig. 2), which was not significantly different from the effect of bafilomycin on TfR surface expression in nontransfected PC12 cells (63.2 $\pm$ 6.8%; data not shown). Surprisingly, DAT exhibited a trend toward increased surface expression in response to bafilomycin A$_1$ treatment (117 $\pm$ 8.0% of vehicle, $n = 4$). Taken together with our 18 °C temperature block results, these data demonstrate that DAT constitutively traffics but suggest that DAT trafficking is mechanistically distinct from that of TfR.

PKC-modulated DAT Trafficking—To test whether intracellular DAT accumulation in response to PKC activation is due to increased DAT internalization, we examined DAT endocytosis
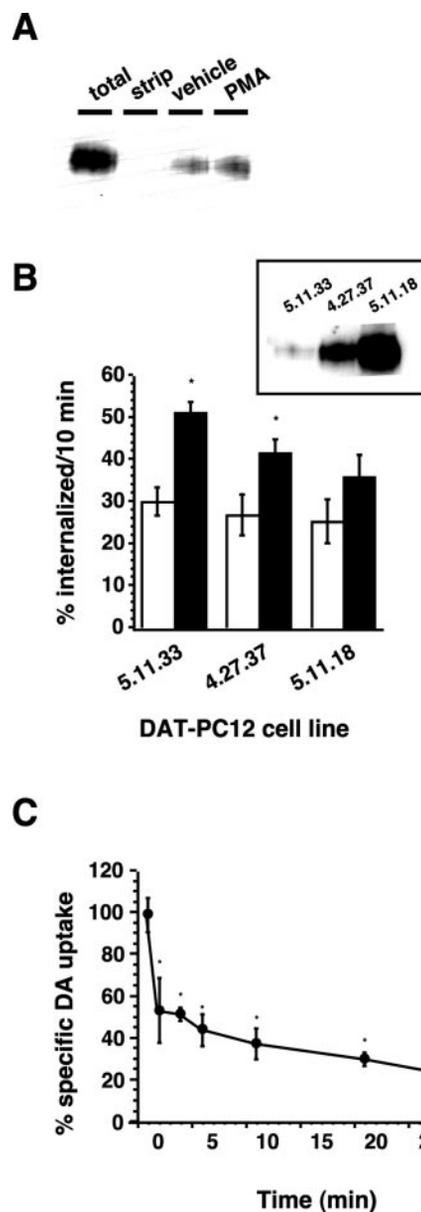

FIG. 3. **Relative DAT internalization rates are sensitive to PKC activation and expression level.** *A*, representative immunoblot showing total cell surface DAT labeled at 0 °C (*total*), MesNa strip of 0 °C biotinylated cells (*strip*), and 10′ internalized DAT $\pm$1 μM PMA. *B*, DAT internalization was assessed at 37 °C in untreated (*open bars*) *versus* 1 μM PMA-treated (*filled bars*) DAT-PC12 cell lines. Data are expressed as % total surface DAT internalized in 10 min $\pm$ S.E. ($n = 2$–11). *, $p < 0.01$ compared with vehicle-treated cells (Student's *t* test, $n = 5$–12). *Inset*, immunoblot of DAT expression in three stably transfected DAT-PC12 cell lines. Equivalent amounts of total cellular protein were loaded. *C*, time course of PMA effect on DA transport. Data are expressed as % specific DA uptake $\pm$ S.E. *, $p < 0.01$ (ANOVA with Dunnett's *post hoc* analysis, $n = 2$).

directly. As endocytic rates for rapidly internalizing proteins are linear for ~10–15 min post-initial internalization (27–29), we used a 10-min internalization assay to determine relative DAT endocytic rates in DAT-PC12 cells (clonal line 4.27.37). As seen in Fig. 3, DAT exhibited robust basal internalization assessed over a time span of 10 min (27.29 $\pm$ 3.4%, $n = 6$), directly demonstrating constitutive DAT endocytosis. Phorbol ester-mediated PKC activation induced a significant increase in the %DAT internalized (43.9 $\pm$ 3.2% $n = 12$), whereas vehicle treatment had no significant effect on DAT internalization (38.9 $\pm$ 6.1%; see Table I). The lack of difference in DAT internalization was not because of a lag in PMA efficacy, as we

TABLE I

Effect of DAT expression level on DAT trafficking in stably transfected PC12 cell lines

Data are expressed as mean ± S.E. Numbers (*n*) in parentheses are the number of independent experiments.

DAT attribute evaluated	DAT-PC12 cell line		
	5.11.33 (*n*)	4.27.37 (*n*)	5.11.18 (*n*)
Relative DAT expression level	0.13	1.00	5.85
Surface expression (% total DAT)	39.5 ± 2.4 (7)	43.3 ± 2.5 (8)	47.1 ± 1.8 (4)
Internalization (% internalized/10 min)			
Basal[a]	30.3 ± 4.9 (6)	27.2 ± 3.4 (6)	25.7 ± 5.2 (5)
Vehicle[a]	25.0 ± 2.0 (2)	38.9 ± 6.1 (7)	12.6 ± 0.4 (2)
1 μM PMA	51.8 ± 3.3[b] (5)	43.9 ± 3.2[b] (12)	36.6 ± 5.6 (6)
Recycling (% biotinylation, 37 °C, 30 min)			
Vehicle[a]	72.7 ± 3.7 (6)	74.5 ± 3.4 (8)	80.6 ± 4.0 (5)
1 μM PMA	60.0 ± 5.1[c] (5)	59.7 ± 2.9[c] (7)	77.7 ± 5.3 (5)

[a] No significant difference among cell lines, ANOVA ($p > 0.05$).

[b] Significantly different from basal, Student's *t* test ($p < 0.01$).

[c] Significantly different from vehicle, Student's *t* test ($p < 0.02$).

detected significant losses in [³H]DA uptake as early as 1 min after PMA addition (Fig. 3*C*).

Given that our stable cell line was derived from a single clone, we asked whether DAT expression levels in our stable PC12 cell line might potentially influence constitutive and regulated DAT endocytosis. To test this possibility, we evaluated DAT trafficking in two other clonal DAT-PC12 cell lines that express 5.8 ± 1.8-fold more (5.11.18 cells, $n = 3$) and 7.8 ± 1.6-fold less (5.11.33 cells, $n = 5$) DAT than the 4.27.37 DAT-PC12 cell line, as assessed by quantitative immunoblot (Fig 3*B, inset*). Cell surface biotinylation revealed that DAT expression level had no effect on the percent total DAT expressed at the plasma membrane (Table I). Similarly, 10-min internalization studies demonstrated that constitutive DAT internalization occurs at the same rate, independently of DAT expression levels (Table I). In cells with the lowest DAT expression level (5.11.33), treatment with 1 μM PMA increased DAT endocytosis from $30.3 \pm 4.9\%$ (basal, $n = 6$) to $51.8 \pm 3.3\%$ (PMA, $n = 5$), consistent with our findings in the moderately expressing 4.27.37 cell line (see above). In striking contrast, cells with high DAT expression levels (5.11.18 cells) exhibited comparable basal DAT endocytosis ($25.7 \pm 5.2\%$, $n = 5$) that did not significantly change in response to PKC activation (Fig. 3*B*, $36.6 \pm 5.6\%$). Taken together, these results demonstrate that DAT constitutively internalizes in a manner that is insensitive to the DAT expression level and that PKC activation enhances DAT endocytosis in a manner sensitive to the DAT expression level.

Although our results demonstrating loss of surface DAT during the 18 °C temperature block strongly suggest that DAT constitutively recycles, direct demonstration of DAT recycling has not been achieved to date. To further investigate DAT recycling, we performed biotinylation at 37 °C with the cell-impermeant biotinylation reagent sulfo-NHS-SS-biotin. We reasoned that if DAT constitutively traffics to and from the plasma membrane, then biotinylation under trafficking-permissive conditions (37 °C) should significantly increase the amount of biotinylated DAT as compared with biotinylation performed under trafficking-restrictive conditions (0 °C). Biotinylation at 37 °C for 45 min was sufficient to biotinylate essentially the entire DAT and TfR pools in 4.27.37 DAT-PC12 cells (Fig. 4*A*). Under these conditions, the early endosomal marker rab5A was not biotinylated nor was there an increase in sodium/potassium ATPase biotinylation at 37 *versus* 0 °C (not shown), demonstrating the specificity of the 37 °C biotinylation for constitutively trafficking proteins. We next performed 37 °C biotinylation to investigate the effect of acute PKC activation on DAT recycling in the three DAT-PC12 cell lines. Both DAT and TfR robustly recycled to the plasma membrane independently of DAT expression level (Fig. 4, *C* and *D*), and PKC activation had no effect on TfR recycling
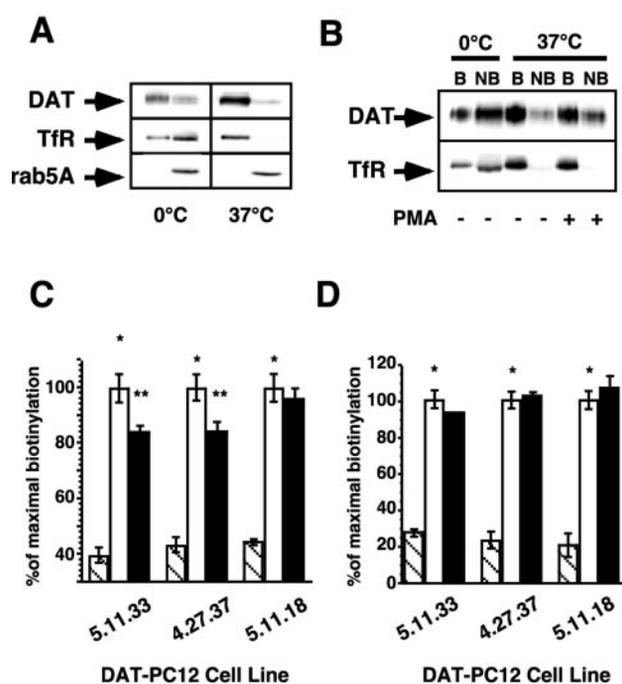

FIG. 4. **DAT constitutively recycles and PKC decreases DAT recycling rates.** *A*, biotinylation for 45 min at 4 and 37 °C. Biotinylated (*B*) and nonbiotinylated (*NB*) proteins were separated by streptavidin pull-down and assessed by immunoblot for DAT, TfR, and the early endosomal marker rab5A. Similar results were obtained in three independent experiments. *B*, DAT-PC12 cells were biotinylated for 30 min at either 0 or 37 °C in the presence of vehicle or 1 μM PMA. Biotinylated and nonbiotinylated proteins were separated by streptavidin pull-down. A representative blot is shown. *C*, averaged DAT recycling data from 0 °C (*hatched bars*) *versus* 37 °C biotinylation in the presence of either vehicle (*open bars*) or 1 μM PMA (*filled bars*). Data are expressed as % maximal biotinylated protein ± S.E. for each cell line. *, $p < 0.001$ compared with 0 °C biotinylation (ANOVA with Bonferroni multiple comparisons test, $n = 5$–8); **, $p < 0.05$ compared with 37 °C vehicle treatment (ANOVA with Bonferroni multiple comparisons test, $n = 5$–8). *D*, averaged TfR recycling data from 0 °C (*hatched bars*) *versus* 37 °C biotinylation in the presence of either vehicle (*open bars*) or 1 μM PMA (*filled bars*). Data are expressed as % maximal biotinylated protein ± S.E. *, $p < 0.01$ compared with 0 °C biotinylation (ANOVA with Bonferroni multiple comparisons test, $n = 2$–8).

(Fig. 4*D*). However, PMA treatment significantly reduced DAT recycling in low and moderately expressing DAT-PC12 cells, whereas DAT recycling was not reduced in the high expressing cell line. These results suggest that DAT constitutively recycles in a manner independent of expression level and that PKC activation slows DAT recycling back to the plasma membrane.

DISCUSSION

Recent reports indicate that membrane trafficking plays a pivotal role in several forms of central nervous system plasticity (30–33). Despite previous results demonstrating that membrane trafficking mediates PKC-induced DAT down-regulation (20, 21), the mechanisms underlying these regulatory events are unknown. Here, we demonstrate that DAT robustly internalizes and recycles to the plasma membrane in PC12 cells and that PKC-induced DAT sequestration is achieved by a combination of increased DAT internalization and decreased DAT recycling. Similar PKC effects on both internalization and recycling have recently been described for E-cadherin (34). Modulation of recycling rates is a mechanism known to occur for other neuronal proteins, including transporters. For example, γ-aminobutyric acid receptor$_A$ (35) and neuronal Na^+/H^+ exchanger (36) constitutively traffic but are retained intracellularly by attenuating recycling in response to PKC activation and phosphatidylinositol 3-kinase inhibition, respectively. Although the results in any expression system must be considered carefully, it is likely that DAT trafficking in PC12 cells reflects neuronal DAT trafficking, as many neuronal proteins traffic faithfully in PC12 cells as they do in neurons, including the m4 muscarinic receptor (37–39), the neuronal Na^+/H^+ exchanger (40), and the nerve growth factor receptor TrKA (41). Moreover, our findings on constitutive DAT trafficking are consistent with recent reports demonstrating that GAT1 internalizes and recycles rapidly both in hippocampal neurons (42) and heterologous expression systems (43), suggesting that transporter trafficking may be a fundamental process occurring across the GAT1/NET gene family.

Many plasma membrane proteins are static in the plasma membrane but internalize in response to activated cellular signaling. For example, G-protein-coupled receptors exhibit very little basal endocytosis (44, 45) but rapidly internalize during agonist-induced desensitization (46, 47). In contrast, proteins such as TfR (48), furin (49), and γ-aminobutyric acid receptor$_A$ (35) are constitutively trafficked and can be regulated at multiple endocytic stages. To examine whether DAT constitutively traffics, we imposed two mechanistically independent recycling blockades on DAT-PC12 cells: first, an 18 °C temperature block and, second, treatment with the antibiotic bafilomycin A_1. We observed significant losses in both DA transport and DAT surface expression when DAT-PC12 cells were incubated at 18 °C for 30 min (Fig. 1). Interestingly, DAT losses from the cell surface were not significantly different from those observed for TfR (Fig. 1*B*), a protein that undergoes constitutive endocytosis at a rate of ~10%/min (48), suggesting that basal DAT internalization rates may approach those of TfR. DAT losses from the plasma membrane were not due to blocking the exit of newly synthesized DAT from the trans-Golgi network, as cycloheximide treatment did not alter either DAT surface expression or total DAT levels in a 3-h time frame, consistent with results from Daniels and Amara (20) examining the basal stability of green fluorescent protein-tagged DAT in cycloheximide-treated Madin-Darby canine kidney cells. Therefore, DAT surface losses at 18 °C reflect robust basal DAT endocytosis, supporting the hypothesis that constitutive recycling is necessary to maintain steady state DAT surface levels.

An acidic endosomal lumen is necessary for most proteins to progress past the early endosome. Bafilomycin potently inhibits the vacuolar ATPase, thereby raising the endosomal pH and arresting endocytic trafficking (50, 51). Indeed, A 30-min bafilomycin treatment significantly reduced TfR surface expression in both PC12 and DAT-PC12 cells (Fig. 2), consistent with the findings of Presley *et al.* (26). Surprisingly, we observed a trend toward increased DAT surface expression fol-
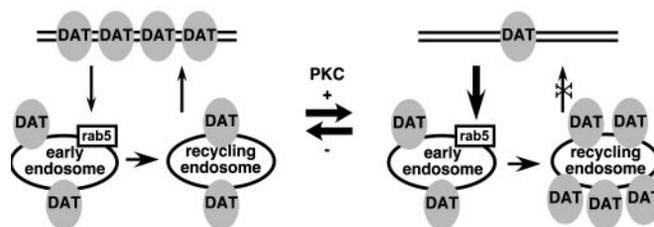

FIG. 5. **Model for constitutive and PKC-regulated DAT trafficking.** Dynamic DAT trafficking is a rheostat that fine-tunes DA signaling. Under basal conditions DAT continually cycles through the endocytic pathway. Upon PKC activation, DAT internalization is accelerated, and DAT recycling back to the cell surface is attenuated. Thus, DAT accumulates in recycling endosomes, and DAT surface pools are depleted.

lowing bafilomycin treatment, suggesting that early endocytic trafficking of DAT and TfR are mechanistically distinct. DAT may reside in a subpopulation of early endosomes that is relatively insensitive to bafilomycin and/or pH changes such as reported for cellubrevin (52). Alternatively, DAT may be intrinsically less dependent upon pH for endosomal sorting. Interestingly, Chinni and Shisheva (53) observed bafilomycin-induced GLUT4 translocation to the plasma membrane, similar to the actions of insulin but downstream of phosphatidylinositol 3-kinase activity. Both DAT (54) and NET (55) are similar to GLUT4, in that they exhibit phosphatidylinositol 3-kinase-dependent, insulin-induced increases in surface expression. Given our results that bafilomycin does not decrease, and may increase, DAT surface expression, it is tempting to speculate that DAT and GLUT4 recycling may share common mechanisms. Future studies aimed at elucidating the mechanisms underlying DAT endocytic trafficking should be illuminating in this regard.

Given our results that DAT constitutively internalizes and recycles, we investigated whether PKC increases DAT internalization, decreases DAT recycling or both. DAT internalized basally at ~30% in 10 min, which translates into a plasma membrane $t_{1/2} \sim 13$ min. These results, consistent with our findings demonstrating 18 °C-induced DAT surface losses, directly confirm basal DAT internalization. PKC activation did not significantly change the endocytic rate of DAT when DAT was expressed at high levels but markedly increased DAT endocytosis when DAT was expressed at low and moderate levels (Fig. 3*B*). Interestingly, although regulated DAT trafficking was sensitive to DAT expression levels, constitutive DAT trafficking was essentially identical across the three cell lines used, representing a >45-fold range in DAT protein levels. These results raise the possibility that constitutive and regulated DAT trafficking occur via distinct cellular mechanisms, consistent with the differential trafficking saturation levels observed for other membrane proteins. For example, β-adrenergic receptor overexpression saturates its trafficking but not that of the TfR (56). Similarly, TfR, low-density lipoprotein receptor and epidermal growth factor receptor expression at levels sufficient to saturate endocytosis for an individual receptor do not saturate the endocytic capacity of another receptor, consistent with the concept of distinct endocytic pathways and/or cofactors for these three receptors (57).

We previously demonstrated DAT enrichment in the TfR-positive recycling endosomes under basal conditions and DAT translocation to recycling endosomes during PKC stimulation (21). Basal DAT enrichment in recycling endosomes strongly suggests that DAT constitutively recycles to the cell surface. To directly test whether DAT recycles, we compared DAT biotinylation at 37 *versus* 0 °C using the cell-impermeant biotinylation reagent sulfo-NHS-SS-biotin. When DAT-PC12 cells were

biotinylated at trafficking-restrictive temperatures (0 °C), we consistently observed 43.3 ± 2.5% of total DAT on the cell surface (Table I). We predicted that if we biotinylated DAT-PC12 cells under trafficking-permissive conditions (37 °C), recycling proteins would gain access to the biotinylation reagent as they reached the plasma membrane and we would observe significant increases in the percent total biotinylation. Indeed, we observed complete biotinylation of both TfR and DAT after 45 min at 37 °C (Fig. 4*A*), suggesting that DAT recycles to the plasma membrane and does so in a time frame comparable with that of the TfR. Biotinylation at 37 °C was restricted to constitutively trafficking proteins, as the early endosomal marker rab5A was not biotinylated at 37 °C (Fig. 4*A*) nor was Na$^+$/K$^+$ ATPase biotinylation increased (data not shown).

Our results demonstrating DAT recycling differ from those of Daniels and Amara (20), who detected lysosomal degradation of green fluorescent protein-tagged DAT during phorbol ester-mediated PKC stimulation. These differences likely reflect either PMA treatment times (30 min *versus* 1–4 h), the cell type in which DAT was expressed (PC12 *versus* Madin-Darby canine kidney cells), or the DAT form studied (wild type *versus* green fluorescent protein-tagged). In striking contrast, when DAT-PC12 cells were biotinylated at 37 °C for 30 min in the presence of 1 μM PMA, DAT biotinylation significantly decreased as compared with vehicle-treated cells in low and moderate expression levels with no loss of total DAT. TfR recycling was unaffected by PMA, suggesting that PMA does not have pleiotropic effects on membrane trafficking. Although we cannot completely rule out the possibility that DAT exit rates from the early endosome are not affected by PKC, our results suggest that decreased DAT recycling causes a "back-up" of DAT in the recycling endosome, consistent with our previous results describing PKC-mediated DAT translocation to recycling endosomes (21) (see model in Fig. 5). Interestingly, the altered trafficking rates we detected do not completely account for the rate at which DA transport activity is lost (Fig. 3*C*), suggesting that PKC may also intrinsically inactivate DAT in addition to altering DAT trafficking kinetics. Although we cannot completely rule out the possibility that DAT trafficking and activity may be modulated by other signaling molecules stimulated by the phorbol ester treatment, our previous work (21) and reports from others (58–61) suggest that phorbol ester-mediated DAT down-regulation occurs primarily via PKC activation. Future studies examining endocytic-defective DAT point mutants should distinguish between intrinsic and trafficking-mediated DAT inactivation mechanisms.

What are the molecular factors governing constitutive DAT trafficking? The DAT primary amino acid sequence codes for several candidate dileucine motifs with potential to drive DAT endocytosis. However, the specific DAT endocytic motif is not defined currently. Phosphorylation is also linked to DAT trafficking, as both PKC activation and protein phosphatase inhibition increase DAT phosphorylation levels (59, 60). Proteolytic digestion (62) and truncation approaches (63) demonstrate that PKC increases in DAT phosphorylation are targeted to DAT N-terminal domains, whereas site-directed mutagenesis suggests that more distal residues are required for PKC mediated DAT phosphorylation (64). However, PKC-induced DAT sequestration still occurs in the absence of increased DAT phosphorylation, suggesting complex relationships among DAT trafficking, phosphorylation, and regulation. Recent evidence supports the hypothesis that transporter protein-protein interactions are prominent in transporter trafficking and function. For example, syntaxin 1A modulates GAT1 (65, 66), NET (67), and SERT (68) trafficking and intrinsic activity. Additionally, DAT, SERT, and NET exist in regulated complexes with pro-

tein phosphatases (69), and DAT complexes containing the PDZ-binding protein PICK1 (70) and the adaptor protein Hic-5 (71) have been observed in neurons and expression systems.

Why would neurons expend energy to constantly cycle transporters? Constitutive trafficking, perhaps best described for the ionotropic glutamate receptors in dendritic spines (31, 72, 73), is emerging as a mechanism employed by neurons to prime key signaling proteins for rapid redistribution. Likewise, transporters play a key role in synaptic strength and are likely to require a dynamically primed environment. Rather than remaining static and waiting for input to initiate trafficking, constitutive DAT cycling operates much like a rheostat, capable of quickly and efficiently fine-tuning DA neurotransmission (for model, see Fig. 5).

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The Dopamine Transporter Constitutively Internalizes and Recycles in a Protein Kinase C-regulated Manner in Stably Transfected PC12 Cell Lines

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