Regulated Trafficking of the Na^+- and Cl^--dependent Neurotransmitter Transporters

By

Gwynn Marie Daniels

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Gwynn Marie Daniels

has been approved

[redacted]

Professor in charge of thesis

[redacted]

Member

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Member

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Associate Dean for Graduate Studies

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Abstract

The reuptake of neurotransmitters through high affinity Na^+-dependent plasma membrane transporters is essential to normal synaptic signaling. Accumulation of released neurotransmitter back into the presynaptic neuron replenishes neurotransmitter stores, and limits the activation of presynaptic and postsynaptic receptors. Inhibition of neurotransmitter transporters elevates extracellular neurotransmitter levels, increasing the intensity and duration of receptor activation. The importance of transporter function is evidenced by the profound behavioral and physiological changes produced by drugs that block neurotransmitter transporters. Therefore, studies of transporter function and regulation may have pathological and therapeutic significance.

To better understand how the activity of the Na^+- and Cl^--dependent neurotransmitter transporters is regulated, we have examined the effect of protein kinase activation on transporter function. Transport activity is inhibited by 40-60% when MDCK cells expressing the dopamine transporter, norepinephrine transporter or γ-aminobutyric acid transporter type 3 are exposed to phorbol 12-myristate 13-acetate (PMA), a potent activator of protein kinase C (PKC). Inhibition of the transporters by PMA is time- and concentration-dependent, and is blocked by the PKC inhibitor staurosporine. In contrast, activators of the cAMP-dependent protein kinase (PKA) pathway have no effect on transporter function. PMA-mediated inhibition of transport activity results from a decrease in the maximal velocity of transport, with no change in the apparent affinity of the transporters for substrate. Biotin labeling of surface molecules revealed a loss of transporter protein from the cell surface following stimulation with PMA. The distribution of the carriers was also examined by indirect immunofluorescence. Under normal conditions, the transporters are primarily localized to the plasma membrane. However, following exposure to PMA, the majority of the

transporter protein is detected in vesicles within the cytoplasm. Confirming the results obtained with functional assays, transporter localization is not effected by PKA activators, and PMA-mediated internalization is blocked by staurosporine.

To facilitate our examination of PKC-mediated alterations in the trafficking of the neurotransmitter transporters, we used a green fluorescent protein-tagged dopamine transporter (GFP:DAT) expressed in MDCK cells. The use of a fluorescent tag allowed us to follow the movement of the dopamine transporter in live cells using confocal microscopy. Over time, transporter molecules are redistributed from the plasma membrane to subcellular vesicles following exposure to PMA. Translocation of the GFP:DAT is independent of the presence of dopamine or cocaine. The GFP:DAT is internalized by an increase in clathrin-mediated endocytosis, that is dependent on the activity of the GTPase dynamin 1. GFP:DAT is first internalized into early endosomes where it colocalizes with the transferrin receptor. Subsequently, GFP:DAT enters the lysosomal pathway, and is targeted for degradation. In the absence of *de novo* protein synthesis, GFP:DAT is fully degraded within 2 hours of PKC stimulation.

The results of these experiments indicate that the Na+/Cl- transporters are acutely regulated by activation of PKC. Inhibition of transport activity results from alterations in the cellular trafficking of the transporter protein. Kinase-mediated changes in the trafficking of neurotransmitter transporters may play a role in modulating synaptic transmission in the central nervous system.

Functional Regulation of the Na^+- and Cl^--dependent Neurotransmitter Transporters by Protein Kinases

Gwynn M. Daniels‡¶ and Susan G. Amara‡§¶

From the ‡Department of Cell and Developmental Biology, §Howard Hughes Medical Institute, and ¶Vollum Institute for Advanced Biomedical Research, Oregon Health Science University, Portland, Oregon 97201.

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The Na^+/Cl^--dependent Neurotransmitter Transporter Family *

Synaptic neurotransmission is achieved through the coordination of four distinct phases. In response to an action potential, neurotransmitter molecules are released from vesicular stores through calcium-dependent exocytosis into the synaptic cleft. Free neurotransmitter in the extracellular space binds cognate receptors on the postsynaptic cell and autoreceptors on the presynaptic neuron mediating signal transduction. Neurotransmitter molecules are removed from the synaptic cleft by accumulation into the presynaptic neuron or surrounding glia through the action of high affinity plasma membrane reuptake systems. The cycle is completed by the actions of vesicular transporters that mediate the translocation of neurotransmitter from the cytoplasm into synaptic vesicles where it becomes available for additional rounds of release (see Fig. 1).

Plasma membrane neurotransmitter transporters play an essential role in both the termination of synaptic transmission and the recycling of neurotransmitters. The spatial and temporal control of synaptic neurotransmission is achieved by maintaining a balance between the release and reuptake of neurotransmitter molecules. Inhibition of the neurotransmitter transporters raises extracellular concentration of neurotransmitters, which can then activate receptors for longer duration and over greater distances than normal. Stimulation of transport activity also modulates synaptic strength by reducing

* The abbreviations used are: GABA, γ-aminobutyric acid; GAT, GABA transporter; DAT, dopamine transporter; SERT, serotonin transporter; GLYT, glycine transporter; PROT, proline transporter; BGT, betaine/GABA transporter; TAUT, taurine transporter; CREAT, creatine transporter; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PKG, cyclic GMP-dependent protein kinase; NO, nitric oxide; CamKII, calcium/calmodulin-dependent protein kinase II; PTK, protein tyrosine kinase; OA, okadaic acid; mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; mGluR, metabotropic glutamate receptor; CTX, cholera toxin;IL-1β, interleukin-1β; EGF, epidermal growth factor; MDCK, Madin Darby canine kidney; GFP, green fluorescent protein; CCP, clathrin coated pit.

Figure 1. **Neurotransmitter Transporters at the Synapse.** Neurotransmitter is released from synaptic vesicles and binds cognate receptors on the presynaptic and postsynaptic cells. Neurotransmitter is then translocated from the extracellular space into the presynaptic cell and surrounding glia by the action of the Na^+/Cl^--dependent neurotransmitter transporters. The accumulation of neurotransmitter is energetically coupled to the flow of sodium ions moving down their concentration gradient, which is maintained by the Na^+/K^+-ATPase. The transport of neurotransmitter is also coupled to the flow of additional ions, such as chloride and potassium. Neurotransmitter from the cytoplasm is concentrated into synaptic vesicles by the action of vesicular transporters.

the amount of neurotransmitter available at the synapse, thereby limiting its effects on presynaptic and postsynaptic receptors.

The plasma membrane neurotransmitter transporters use the movement of ions down their electrochemical gradient to drive the movement of substrate against its concentration gradient. In particular, translocation of neurotransmitter across the plasma membrane is energetically coupled to the electrochemical gradient of sodium, which is maintained by the Na^+/K^+-ATPase. The accumulation of neurotransmitter is also coupled to the flow of additional ions, for example the cotransport of chloride ions or the countertransport of potassium ions.

The presence of distinct high affinity, sodium-dependent reuptake systems was first described by Axelrod et. al. forty years ago (10). We now know that the plasma membrane neurotransmitter transporters are represented by two distinct gene families. The Na^+- and Cl^--dependent family of transporters, which are characterized by the cotransport of chloride ions in addition to the cotransport of sodium ions, includes the high affinity carriers for the neurotransmitters dopamine, norepinephrine, serotonin, γ-aminobutyric acid (GABA), and glycine. This family also includes transporters specific for the accumulation of the neuromodulatory amino acid proline, the osmolytes betaine and taurine, and creatine, which plays an essential role in energy storage in muscle. A separate family of transporters allows for the reuptake of the excitatory amino acids glutamate and aspartate, as well as the neutral amino acids alanine, serine and cysteine. Both families of neurotransmitter transporters are characterized by their absolute dependence on the presence of sodium ions. However, the excitatory amino acid transporters are distinguishable from the Na^+/Cl^--dependent transporters by sequence homology, pharmacology, protein structure, and dependence on ions other than Na^+.

Molecular cloning of the Na^+/Cl^--dependent Neurotransmitter Transporters

The *cis*-3-aminocyclohexanecarboxylic acid (ACHC)- and nipecotic acid-sensitive GABA transporter (GAT1) was the first of the Na^+/Cl^--dependent neurotransmitter transporters to be cloned (58), following purification of the protein to apparent homogeneity (140,141). GAT1 is primarily localized to the terminals of GABAergic neurons (107), and is now known to represent one of four subtypes of GABA transporters that vary by amino acid sequence, inhibitor sensitivity and tissue distribution. The GAT2 is sensitive to β-alanine, and is expressed in non-neuronal cells in the brain, and in the retina and kidney (97). GAT3, which is expressed in both neurons and glia, is sensitive to both β-alanine and nipecotic acid and is also expressed in the kidney (35). GAT4 is inhibited by nipecotic acid and is primarily restricted to the brain (94).

Expression cloning of the norepinephrine transporter (NET) (125) revealed a striking sequence similarity with the GABA transporter GAT1, first defining them as members of the same gene family. Homology screening identified cDNAs for the closely related dopamine transporter (DAT) (50,79,160), and serotonin transporter (SERT) (22,67). The distribution of the monoamine transporters within the central nervous system (CNS) is consistent with their presynaptic localization. The NET shows restricted expression only in noradrenergic neurons, the DAT in dopaminergic neurons, and the SERT in serotonergic neurons. In addition, the NET and SERT are expressed at high levels in the adrenal medulla. There is a certain level of overlap in the inhibitor sensitivity of the monoamine transporters (57) presumably related to their high degree of sequence identity.

The glycine and proline transporters are also members of the Na^+/Cl^--dependent neurotransmitter transporter family. Two subtypes of glycine transporter (GLYT) have been identified. GLYT1, which is sarcosine sensitive, is expressed primarily in glial cells in the brain stem and spinal cord, and to a lesser degree in some brain regions (166).

GLYT2, on the other hand, is insensitive to sarcosine, and is localized to only to glycinergic neurons of the spinal cord, brainstem and cerebellum (93). The proline transporter (PROT) is expressed in a subpopulation of glutamatergic neurons, where it is believed to play a neuromodulatory role (44).

Other members of the Na^+/Cl^- transporter family that have been cloned include the transporters for betaine, taurine, and creatine. The betaine/GABA transporter (BGT) and taurine transporter (TAUT) are expressed at high levels in the kidney, where they function as osmoregulators (176,184). Creatine serves as a major source of energy storage in muscle, and the creatine transporter (CREAT) is expressed primarily in the heart and skeletal muscle (59). The TAUT and CREAT are also expressed in the brain, although their precise function in the CNS remains unclear.

Common Structural Motifs

The cloning of the Na^+/Cl^--dependent transporters, and elucidation of their primary amino acid sequence revealed not only striking sequence homology, but the presence of shared structural elements as well (Fig. 2). Hydropathy analysis of the cloned transporters indicates the presence of 12 hydrophobic regions that are believed to represent membrane spanning domains. The absence of a signal sequence suggests that the amino terminus resides within the cytoplasm, and the presence of an even number of transmembrane spanning regions implies that the carboxyl terminus has an intracellular localization as well. The putative transmembrane domains are connected by rather short hydrophilic loops, with the exception of a large loop between transmembrane domains 3 and 4, which is predicted to be extracellular. The intracellular localization of both the N- and C-termini, as well as the orientation of the large loop on the extracellular face of the plasma membrane were confirmed for both the dopamine and norepinephrine

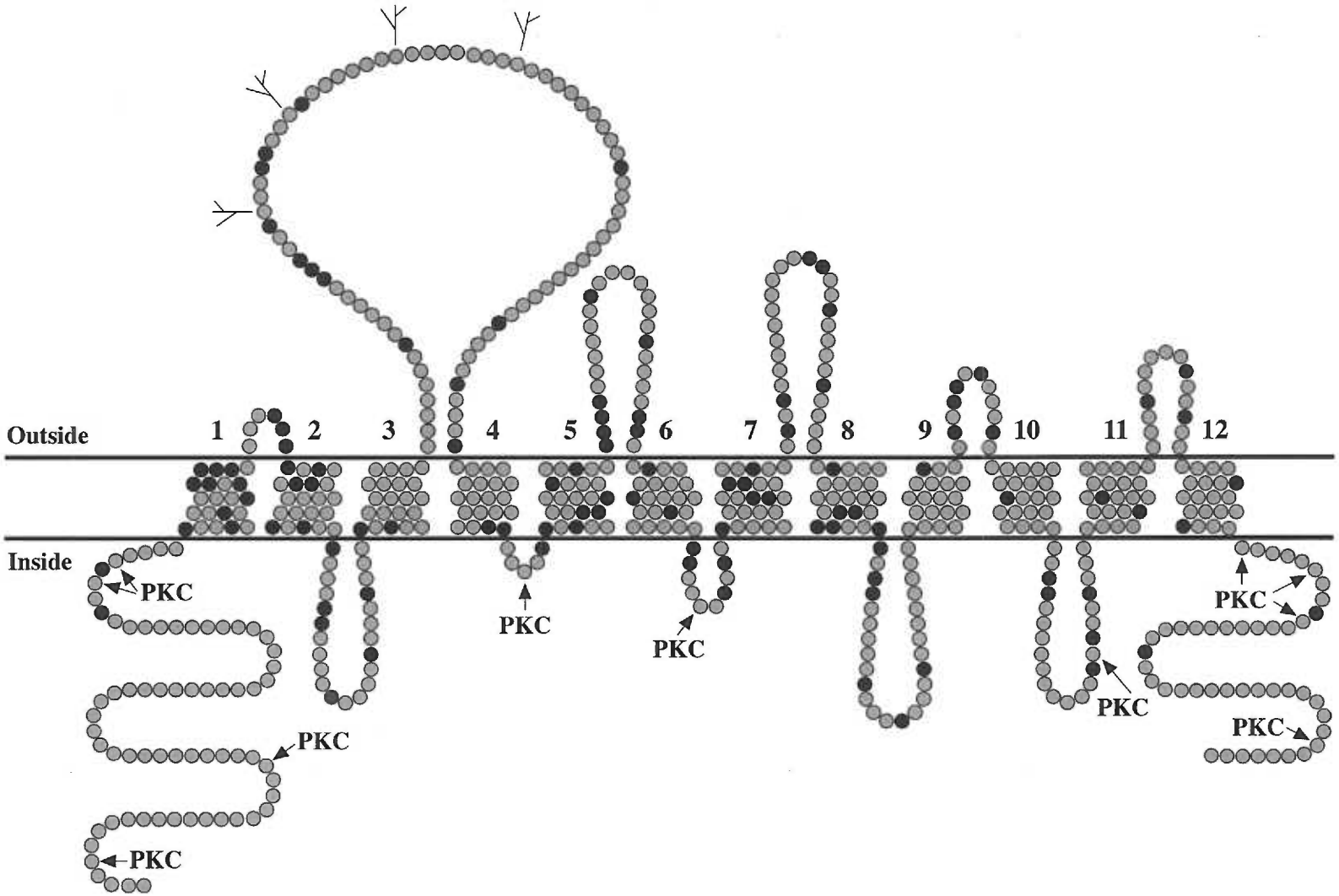
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Figure 2. **Model of Na^+/Cl^- Transporter Structure.** Representation of the putative topology and structural features of the Na^+/Cl^--dependent neurotransmitter transporters. As suggested by the primary sequence, the proteins have 12 transmembrane domains with both the amino and carboxyl termini oriented intracellularly. The large extracellular loop between TM3 and TM4 contains 2-4 consensus sites for N-linked glycosylation. Depicted is the rat dopamine transporter indicating canonical sites for PKC phosphorylation. Dark circles designate amino acid residues which are absolutely conserved between the rat homologs of the DAT, GAT1, GAT3, GLYT1, NET, SERT, PROT, TAUT, CREAT, and the canine BGT.

transporters by the use of antisera specific to intracellular or extracellular epitopes (27,64). More detailed studies of the membrane topology of GAT1 (18,34) indicate that while the early models proposed for the structure of these molecules is basically correct, their topology is somewhat more complex than originally proposed.

The primary amino acid sequence of the Na^+/Cl^- transporters also reveals consensus sites for post-translational modifications. The large extracellular loop found between transmembrane domains 3 and 4 contains multiple sites for N-linked glycosylation in all of the carriers. The importance of glycosylation to transporter function primarily involves protein trafficking. Mutants lacking consensus glycosylation sites are unaltered in their affinity for substrate and inhibitor binding, however their ability to accumulate neurotransmitter is significantly reduced (102,119,170). The loss in activity of glycosylation mutants has been attributed to a reduction in the amount of transporter protein at the cell surface.

The Na^+/Cl^- transporters also have multiple consensus sites for phosphorylation by protein kinases. These sequences occur primarily within the amino- and carboxyl-terminal tails, although they are also located within the intracellular loops between transmembrane domains. These regions contain canonical recognition sites for cyclic AMP-dependent protein kinase (PKA), protein kinase C (PKC), and calcium/calmodulin-dependent protein kinases.

Pharmacological Significance of Na^+/Cl^--dependent Neurotransmitter Transporters

Drugs that inhibit the monoamine transporters have profound behavioral and physiological consequences, suggesting that endogenous regulation of transport activity may play an important role in normal behavior. The SERT is potently inhibited by many antidepressant drugs, including fluoxetine (Prozac™), paroxetine, sertraline, and citalopram (see 45). The serotonin-specific reuptake inhibitors (SSRIs) are used to treat

a wide spectrum of affective disorders, including major depression, anxiety disorders, and obsessive-compulsive disorders. The classic tricyclic antidepressants amitriptyline and imipramine are also powerful antagonists of the SERT, and of the NET. Desipramine, another of the tricyclic antidepressants, is a selective inhibitor of the NET. The SERT inhibitor fenfluramine is also effective as an appetite suppressant. In addition to the monoamine transporters, the GABA transporters are the targets of clinically important drugs. Antiepileptic drugs, such as tiagabine, are inhibitors of GABA uptake (84), prompting the development of designer drugs that block GABA transporters as antiseizure agents.

Psychomotor stimulants, such as amphetamine and cocaine, nonselectively inhibit the DAT, NET, and SERT (see 5). While these drugs often produce euphoric and stimulant effects, they are ineffective as antidepressants. Another substrate of the DAT is the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) that causes selective loss of nigrostriatal dopaminergic neurons and provides an experimental model for Parkinson's disease (167).

Neurotransmitter transporters may also be implicated in neurological and psychiatric disorders. Changes in the level of DAT binding sites have been associated with Parkinson's disease, Tourette's syndrome, and progressive supranuclear palsy (33,120,164). GABA neurotransmission is altered in schizophrenia, epilepsy, and Huntington's chorea (16,128,148), and variations in SERT activity have been observed in association with anxiety, depression, suicide and autism (104,124).

Targeted Disruption of Na^+/Cl^- Transporter Genes

The recent development of transgenic mice lacking neurotransmitter transporters is providing important insight into their functional significance *in vivo*. As the targets of many drugs with therapeutic and abuse potential, the DAT and SERT have been the focus

of early investigations.

The importance of DAT function has been illustrated by the generation of transgenic mice in which the gene for the DAT has been deleted. The striking increase in spontaneous hyperlocomotion exhibited by DAT knockout mice mimics the behavior elicited by psychostimulants (51). Moreover, DAT knockout mice show no response to the administration of either cocaine or amphetamine. Inhibition of DAT activity has long been believed to underlie the addictive effects of cocaine, by increasing dopamine concentrations in the nucleus accumbens and related targets in the mesolimbic system (149). However, DAT knockout mice continue to self-administer cocaine (150), suggesting that additional sites of action, perhaps the NET or SERT, also play a role in the reinforcing properties of cocaine.

Besides the expected increase in the persistence of extracellular dopamine, loss of the DAT also has many effects on dopamine signaling and homeostasis. While levels of tyrosine hydroxylase, the rate-limiting enzyme in dopamine biosynthesis, are reduced by 90% in DAT knockout mice, synthesis of dopamine is actually twice that of wild type controls (75). However, because of a significant increase in dopamine degradation, overall dopamine concentrations are drastically reduced in homozygous mutants. $DAT^{-/-}$ animals also exhibit a significant decrease in the expression of D_1 and D_2 dopamine receptors (51), suggesting that increased extracellular dopamine has an inhibitory effect on dopamine signaling. The loss of the lactotroph dopamine receptors has a profound effect on pituitary function, leading to inhibited growth and inability to lactate (24).

In spite of its importance as a target of antidepressant drugs, disruption of the SERT gene has little apparent effect on behavior (17). However, $SERT^{-/-}$ mice are resistant to the stimulatory effects of 3,4-methylenedioxymethamphetamine (ecstasy). In parallel with the findings for targeted disruption of the DAT, SERT knockout mice exhibit drastically reduced brain serotonin levels, suggesting that serotonin homeostasis is

affected by the decrease in serotonin clearance. Although a detailed examination of serotonin metabolism has not been completed in SERT knockout mice, such studies are of great significance because of the widespread use of antidepressant medications that inhibit SERT activity.

Regulation of the Na^+/Cl^--dependent Neurotransmitter Transporters

The regulation of transport activity is thought to constitute an important mechanism for the control of neurotransmitter action. Modulation of the transporters can significantly alter synaptic efficacy and may play an important role in synaptic plasticity and learning processes. The activity of the Na^+/Cl^--dependent neurotransmitter transporters is regulated through two distinct mechanisms. Acute regulation occurs very rapidly following the application of an appropriate stimulus and is the result of post-translational modification of existing transporter protein. In contrast, chronic regulation of the transporters occurs over a period of several hours or days and involves changes in transporter mRNA levels.

Regulation by Protein Kinase C

Our most detailed understanding of acute regulation of the Na^+- and Cl^--dependent neurotransmitter transporters comes from studies investigating the effect of PKC activation on transporter function. Activation of PKC by phorbol esters modulates the activity of all members of the Na^+/Cl^--dependent neurotransmitter transporter family examined to date (refer to table 1 for references). Protein kinase activators and inhibitors have been shown to influence transporter function by binding directly to the carrier (62), however the specificity of PKC activation to regulation of transporter function by phorbol esters has been well documented. The inactive α isomers of phorbol esters have no effect on either the activity (6,9,14,25,38,39,52,54,61,70,123,137,138,147,151-153,180,186,189) or the phosphorylation state (70) of the transporters. Furthermore, phorbol 12-myristate 13-acetate (PMA)-mediated regulation of the transporters is effectively blocked by the kinase inhibitor staurosporine (6,8,9,25,38,54,70,81,85,114,123,137,138,147,151-153,180, 186,189), as well as the more PKC-specific inhibitors bisindoylmaleimide (39,147, 180,

Table 1. **Regulation of Na^+/Cl^--dependent Neurotransmitter Transporters by PKC**

NTT	Activator	Cell type/preparation	Response	Reference
rDAT	PMA, OAG	COS	$\downarrow V_{max}$	(81)
rDAT	PMA, DiC_8	synaptosomes*	$\downarrow V_{max}$	(38)
rDAT	PMA, OAG	synaptosomes*	$\downarrow V_{max}$	(180)
hDAT	PMA	oocytes	$\downarrow V_{max}$, $\downarrow B_{max}$, $\downarrow C_m$	(189)
rDAT	PMA	LLC-PK_1	$\downarrow V_{max}$, ↑phosphor.	(70)
hDAT	PMA, PDBu	C6 glioma	$\downarrow V_{max}$, $\downarrow B_{max}$	(186)
hDAT	PMA	Sf9	$\downarrow V_{max}$, ↑internalization	(137)
		COS	↑internalization	
rGAT?	PMA, OAG	neurons*, glia*	↑ K_T	(52)
rGAT1	TPA, OAG	synaptosomes*, oocytes	↑ K_T	(123)
rGAT1	PMA, OAG	oocytes	$\uparrow V_{max}$	(39)
rGAT1	PMA	HEK293	$\downarrow V_{max}$	(153)
rGAT1	PMA	oocytes	$\uparrow V_{max}$, ↑current, $\downarrow C_m$, ↑ at PM, ↓ in vesicles	(139)
rGAT1	PMA	PC12	$\downarrow V_{max}$	(14)
mGLYT1	PMA	HEK293	$\downarrow V_{max}$	(152)
rGLYT1	TPA, OAG	C6 glioma*, COS	↓uptake	(54)
hNET	MCh, PMA	SK-N-SH*	$\downarrow V_{max}$, $\downarrow B_{max}$	(8)
hNET	PMA, PDBu	HEK293	$\downarrow V_{max}$, $\downarrow B_{max}$, ↓surface expression	(9)
		LLC-PK1	$\downarrow V_{max}$, ↑internalization	
		COS-7	$\downarrow V_{max}$	
rb SERT	PMA	lung tissue	$\downarrow V_{max}$, ↑ K_T	(115)
bSERT	PMA, PDBu	endothelial*	↓uptake	(114)
hSERT	TPA, mez	platelets*	$\downarrow V_{max}$	(6,99)
hSERT	PMA, PDBu	HEK293	$\downarrow V_{max}$, ↓current, ↓surface expression	(138)
hSERT	TPA	BeWo*	$\downarrow V_{max}$	(151)
rSERT	TPA	COS-7	$\downarrow V_{max}$	(151)
hSERT	PMA, PDBu	HEK293	↓uptake, ↑phosphor.	(147)
hTAUT	PMA	JAR*	↓uptake	(85)
hTAUT	PMA	HT-29*, CaCo-2*	$\downarrow V_{max}$, $\uparrow K_T$	(25)
hTAUT	PMA	GL15 glioma*	$\downarrow V_{max}$, $\uparrow K_T$	(172)
rTAUT	PMA, OAG	astrocytes*	$\downarrow V_{max}$, $\uparrow K_T$	(173)
mTAUT	PMA	Ehrlich ascites tumor*	↓uptake	(111)
rTAUT	PMA	astrocytes*	↓uptake	(171)
		neurons*, neuroblastoma*	no change	
cTAUT	PMA, PDBu	oocytes	$\downarrow V_{max}$, $\uparrow K_T$	(61)
mTAUT	DOG	oocytes	$\downarrow I_{max}$, $\downarrow Q$, $\downarrow C_m$	(96)

* Native transporter Abbreviations: NTT, neurotransmitter transporter; phosphor., phosphorylation; C_m, membrane capacitance; PM, plasma membrane; mez, mezerein; I_{max}, maximal current; Q, charge transfer.

189), chelerythrine (171,172,173,186), H-7 (52,152,153), and calphostin C (14,61).

Inhibition of the transporters by phorbol esters is both time- and concentration-dependent and has been observed in a variety of cell types and expression systems (refer to Table 1). In spite of the diversity of expression systems that have been used successfully to examine the regulation of the Na^+/Cl^- transporters, the choice of cell type remains an important factor. When expressed in CHO cells for example, the GAT1 is insensitive to regulation by phorbol esters. Cell lines derived from the brain may be more relevant to the study of neurotransmitter transporters, although the heterogeneity in the regulation of the natively expressed transporters in primary cultures of astrocytes and neurons suggests that effects of PMA may be highly cell-type specific (14,173). However, the physiological relevance of PKC-mediated regulation of transport activity is suggested by the strong correlation between the modulation of the transporters expressed in heterologous expression systems and the modulation of native transporters in synaptosomes (DAT, GAT1, SERT), cultured neurons and glia (GAT, TAUT), platelets (SERT), cultured endothelial cells (SERT), Ehrlich ascites tumor cells (TAUT), and cell lines derived from the CNS (GLYT1, NET, TAUT) and periphery (NET, SERT, TAUT).

In addition to having a direct effect on the transporters, PKC activation could modulate transporter activity through indirect actions of the kinase. For example, alterations in ion gradients across the plasma membrane have the capacity to effect the electrochemical driving force of active transport. However, the PMA-mediated inhibition of both the SERT and DAT are independent of extracellular sodium concentration (6,81). While the activity of the Na^+/K^+-ATPase is decreased in response to phorbol esters (20), this appears to be unrelated to inhibition of the transporters, as the presence of oubain, a Na^+/K^+-ATPase inhibitor, does not alter the PMA-mediated inhibition of the transporters (138). Likewise, amiloride, a blocker of the Na^+/H^+ exchanger, has no effect on the downregulation of uptake activity induced by PKC activation (6,123,152,173).

Furthermore, the Na^+-dependent reuptake of leucine, lysine, or alanine, through transport mechanisms distinct from the Na^+/Cl^- transporters, are not altered by the presence of PMA (8,25,70,106,172,173,189). PMA-induced changes in membrane potential might also alter transport activity, by reducing the inward flow of sodium ions. However, the application of PMA does not change the resting potential of either transporter-expressing or control oocytes (189) or transfected HEK 293 cells (152).

The activity of the Na^+/Cl^- transporters is almost universally inhibited by the activation of PKC with phorbol esters. PMA-mediated inhibition of the transporters is generally associated with a decrease in transport velocity (V_{max}), rather than a decrease in the affinity of transporters for substrate reflected by an increase in the Michaelis Menton constant of transport (K_T). However, inhibition of the transporters is sometimes also linked to a small but significant increase in K_T. These results often come from experiments done in tissue preparations or primary cultures, where low expression levels may make accurate K_T determinations difficult (52,115,123). An increase in K_T is observed more often for the TAUT than for the other transporters, suggesting that there may be slight variations in the mechanism of regulation (173).

The stoichiometry of the flow of ions coupled to the translocation of substrate across the plasma membrane, renders the transport process electrogenic. In addition to the current generated by movement of the substrate across the plasma membrane, a transporter-associated "leak" current has also been described for this family of transporters (168). Electrophysiological analysis indicates that both the substrate-mediated transport current (96,139,189) and the transporter-induced leak current (138,189) are decreased in response to phorbol esters.

In contrast to the widely observed inhibition of the Na^+/Cl^- transporters in response to activation of PKC, the activity of the neuronal GABA transporter GAT1 expressed in *Xenopus* oocytes has been reported to be stimulated by exposure to PMA

(39,139). This discrepancy in the effects of PMA appears to be due to the method of PMA application. Stimulation of uptake activity was seen when PMA was injected directly into the cytoplasm of GAT1-expressing oocytes. However, when PKC activators are added to the buffer, oocytes expressing the transporters, including GAT1, have consistently shown an inhibition in uptake activity (61,96,189). While the underlying cause of the contrast in these findings is not well understood, they may represent the activation of a subset of PKC isoforms that are preferentially activated when phorbol esters are introduced directly into the interior of the cells.

Phosphorylation of both the dopamine and serotonin transporters is increased over basal levels in response to activation of PKC (70,147,180). Although transporter phosphorylation and inhibition of transport activity occur over a similar time course and to a similar extent, a direct link between phosphorylation and the inhibition of transporter function has not yet been established. Mutation of the canonical PKC phosphorylation sites in GAT1, GLYT1, or SERT does not abolish the PMA-mediated inhibition of transport activity (39,151,152). However, the ability of PMA to induce the phosphorylation of the mutant transporters is not known. While PKC-mediated phosphorylation may be occurring at noncanonical sites, based on the current state of our understanding, it is equally likely that phosphorylation of the transporters is mediated by an unknown kinase that is itself activated by PKC phosphorylation. Furthermore, the possibility exists that the modulation of the transporters results from PKC-mediated alterations in accessory proteins that bind the carriers and are involved in the regulation of their activity.

The identity of amino acid residues within the transporters that are important to regulation by protein kinases is not well established. While the cytoplasmic carboxyl-terminal tail of the hDAT contains multiple consensus sites for phosphorylation by PKC (50), hDAT mutants lacking the carboxyl-terminal tail show increased sensitivity to PMA

(90). This suggests that not only are the PKC sites within the carboxyl-terminal tail of the hDAT not involved in regulation by PKC, but that the deleted region contains negative regulatory elements that effect PMA-mediated modulation as well. Furthermore, as mentioned in the previous paragraph, mutation of canonical PKC phosphorylation sites within the carriers does not have an effect on their regulation by PMA. Recently, a serine residue within a consensus PKC phosphorylation sequence located on an intracellular loop between transmembrane domains 6 and 7 has been demonstrated to be critical to the PKC-mediated regulation of the TAUT (61). An antibody directed toward this intracellular loop has also been shown to abolish the PMA-mediated inhibition of TAUT activity (61). However, this PKC consensus site is not conserved among the Na^+/Cl^- transporters, suggesting that the importance of this serine residue in PMA-mediated regulation may be unique to the TAUT. A leucine heptad repeat located within the second transmembrane domain of the GAT1 has also been shown to be involved in the regulation of this transporter through interactions with cellular proteins (14,139). While this leucine heptad repeat is also found in other members of the Na^+/Cl^--dependent transporter family, for example the NET and PROT, is it not highly conserved. The lack of conservation in amino acid residues shown to be involved in PMA-mediated modulation suggests that even though regulation by PKC is a common property of the Na^+/Cl^- transporters, it may be occurring through different mechanisms.

Several lines of evidence indicate that the PKC-mediated inhibition of the Na^+/Cl^- transporters results from a change in the subcellular distribution of the carriers. The predominant finding of a decrease in V_{max} with no accompanying decrease in affinity is consistent with a reduction in the number of functional transporters at the cell surface. Furthermore, radioligand binding is decreased in intact cells after treatment with PMA (8,186,189), while cell homogenates showed no change in binding capacity (8,189). This difference in radioligand binding between intact cells and homogenates suggests that

while the overall number of transporter molecules remains constant, the number of carriers available at the surface of the cells declines. The activation of PKC also induces a decrease in membrane capacitance in oocytes expressing the transporters (96,139,189), indicating an increase in endocytosis. Cell surface biotinylation of SERT and NET expressing cells confirmed that the PKC-mediated inhibition of the transporters resulted from a decrease in transporter protein at the surface of the cells (9,138). Furthermore, indirect immunofluorescence has shown redistribution of the DAT and NET from the plasma membrane to an intracellular localization (9,137). Regulation of function through protein translocation appears to be a unifying property of the Na^+/Cl^- transporters. Even the PMA-mediated increase in the activity of GAT1 is related to changes in the amount of GAT1 protein at the cell surface. Injection of PMA causes an increase in the amount of GAT1 protein found in the plasma membrane fraction of oocytes and an accompanying decrease in the vesicular fraction (39,139).

The involvement of accessory proteins in the PKC-mediated regulation of the transporters remains an important area of investigation. Although little is known about the identity of such proteins, the PMA-mediated modulation of GAT1 has been linked to its association with proteins involved in cellular trafficking. While the overall relevance of GAT1 upregulation following direct injection of PMA is questionable, it may provide some important insights into the cellular mechanisms of transporter redistribution. Upregulation of GAT1 activity is most pronounced at low expression levels and is nearly eliminated at high expression levels suggesting that cellular factors involved in regulation are being depleted (39,139). When rat brain mRNAs are coinjected into oocytes expressing high levels of GAT1, the ability of PKC to regulate transporter function is recovered (139). Coexpression of syntaxin1A alone is able to restore the PMA-mediated regulation of GAT1 to the same extent as coexpression of brain mRNAs. Furthermore, botulinum C, which specifically cleaves syntaxin1A, abolishes PMA-mediated alterations

in both the activity and localization of GAT1.

Somewhat surprisingly, syntaxin1A is also associated with the PMA-mediated inhibition of the GAT1. GABA uptake activity is inhibited by PMA in PC12 cells expressing the GAT1 (14). In contrast, GAT1 activity is increased by botulinum toxin C, suggesting that syntaxin1A exerts a negative regulatory effect on the transporter. Furthermore, cleavage of syntaxin1A ameliorates the inhibitory effect of PMA. When the GAT1 is stably expressed in CHO cells it is completely insensitive to the effects of either PMA or botulinum toxin. Expression of syntaxin1A in GAT1-CHO cells decreases GABA uptake activity, confirming its role in the negative regulation of GAT1. However, the presence of syntaxin1A alone does not restore PMA sensitivity. Coexpression of syntaxin1A and its binding partner Munc18 eliminates sensitivity to botulinum toxin and restores PMA-mediated modulation of GAT1, although expression of Munc18 alone has no effect on GAT1 activity or its regulation by PMA. Interestingly, Munc18 is a substrate for PKC, and dissociates from syntaxin1A following phosphorylation. While syntaxin was originally characterized as being involved in vesicle docking and fusion, more recently it has been associated with the regulation of ion channel activity (21,118). The mechanism of ion channel regulation by syntaxin is not well understood, but may involve alterations in membrane trafficking. The complex nature of the relationship between syntaxin1A, Munc18, and PMA in the regulation of GAT1 suggests that syntaxin 1A may be involved in both direct modulation and cellular trafficking of the transporter. The involvement of syntaxin, Munc18, or other cellular proteins, in the PKC-mediated regulation of additional members of the Na^+/Cl^- transporter family remains to be determined.

Regulation by Other Protein Kinases

Although the Na^+/Cl^- transporters contain consensus sites for phosphorylation by PKA, the evidence for PKA-mediated regulation of the activity of the Na^+/Cl^- transporters is conflicting and controversial. The activity of the native DAT is upregulated by stimulation of PKA in both striatal suspensions and cultured hypothalamic cells (12,76). However, PKA activators have no effect on either the activity or phosphorylation state of DAT in striatal synaptosomes (174,180). Furthermore, there was no change in the activity of the cloned DAT in response to either forskolin or cAMP analogs using heterologous expressions systems (38,137,189). Likewise, the NET exhibits no alteration in activity following PKA stimulation (8). The endogenous GAT in synaptosomes is inhibited by PKA activators, but only at very high concentrations, making the physiological relevance of this effect questionable (174). There is conflicting evidence that the activity of the TAUT is regulated by PKA activation. Mollerup and Lambert (111) reported that the endogenous taurine uptake in Ehrlich ascites tumor cells was enhanced following activation of PKA, while Loo, et. al. (96) showed a decrease in TAUT-mediated currents in oocytes expressing the cloned TAUT in the presence of 8-Br-cAMP. Interestingly, while phosphorylation of the SERT expressed in HEK-293 cells is increased by agents that stimulate PKA (147), there is no evidence that SERT activity is altered following PKA activation. The inability to detect changes in the activity of the Na^+/Cl^- transporters in response to PKA activation may be due to the limitations of the experimental techniques employed. When measured by rotating disk electrode voltammetry, which can detect rapid changes in the rate of dopamine clearance, stimulation of PKA elicits a rapid and transient increase in DAT activity (12). The effect of PKA activation on the DAT occurs within one minute of 8-Br-cAMP application, a timeframe that is too rapid to be detected by standard transport assays.

There is increasing evidence that cyclic GMP (cGMP)-dependent protein kinase

(PKG) is involved in transporter regulation. Phosphorylation of the SERT is increased following incubation with 8-pCT-cGMP (147), and the activity of both the DAT and the TAUT are inhibited by the addition of 8-Br-cGMP (25,38). The activation of guanylyl cyclases with nitric oxide (NO) generators also results in inhibition of the DAT in striatal preparations (95,132), and the NET in both primary cultures, and in PC12 cells (77). Although the PKG-mediated inhibition of the Na^+/Cl^- transporters, like PKC-mediated inhibition, is due to a decrease in the V_{max} of transport with no significant change in K_T (25,132), regulation of the transporters through these two pathway appears to be unrelated (8). Compounds that generate NO have also been shown to increase the activity of the Na^+/Cl^- transporters. The activity of the endogenous SERT in RBL 2H3 cells and the endogenous NET in SK-N-SH cells are increased by treatment with the NO donor SNAP (8,106). The discrepancy in the effect of NO-generating drugs on transporter function may arise from differences in the production of NO, which at concentrations over 1 μM produces a variety of effects independent of PKG activation (reviewed in 92). It is also possible that cell-type specific variations in PKG pathways cause differential regulation of the transporters.

Calcium/calmodulin-dependent protein kinases may also play a role in regulating the activity of the Na^+/Cl^- transporters. The activity of the native NET expressed in PC12 cells is enhanced by increasing concentrations of extracellular Ca^{++} (175). In synaptosomes, the activity of both the DAT and SERT are also upregulated by extracellular Ca^{++}, through an increase in V_{max} (177,185). While the particular kinase involved in modulating the transporters is unknown, the Ca^{++}-mediated upregulation of the monoamine transporters is blocked by calmodulin agonists and by inhibitors of both the myosin light chain kinase and the calcium/calmodulin-dependent protein kinase II (CamKII), but is unaffected by PKC inhibitors. The Ca^{++}-mediated increase in NET activity is accompanied by an increase in the B_{max} of desipramine binding, suggesting that

the number of carrier molecules at the cell surface is increased (175). Therefore, regulation of the transporters by CamKII, like regulation by PKC, may involve alterations in the cellular trafficking of the carriers. A peptide containing sequences from the carboxyl-terminal tail of the NET that surround Ser 57 is phosphorylated *in vitro* by purified CamKII (175). To date this is the only evidence for direct phosphorylation of a Na^+/Cl^- transporter sequence by a protein kinase. The CamKII inhibitor KN-93 decreases NET activity in SK-N-SH cells (8). Furthermore, the activity of the SERT and TAUT are inhibited by calmodulin antagonists through a decrease in the V_{max} of transport (72,146,173), indicating that calcium/calmodulin-dependent protein kinases are involved in the regulation of basal transport activity. Calmodulin antagonists also block the PMA-mediated inhibition of the TAUT (173), suggesting a convergence of the PKC and calcium/calmodulin-dependent kinase pathways in the regulation of the Na^+/Cl^- transporters.

The arachidonic acid pathway has also been implicated in the regulation of neurotransmitter transporters. Arachidonic acid inhibits the dopamine uptake in striatal synaptosomes (86) and in C6 glioma cells expressing the DAT (187). The effects of arachidonic acid on the DAT are similar to those seen following activation of PKC. Both the V_{max} of transport and the B_{max} of inhibitor binding decrease, while there is no change in the apparent affinity of the transporter for either substrate or inhibitor (187). Although these findings are similar to those seen upon activation of PKC, they appear to be independent of PKC activation, as the PKC inhibitor staurosporine has no effect on arachidonic acid-mediated inhibition of the transporter.

While the Na^+/Cl^- transporters do not contain consensus sites for phosphorylation by protein tyrosine kinases (PTKs), there is some recent evidence suggesting that tyrosine kinases may be involved in the acute regulation of transporters. The nonspecific protein tyrosine kinase inhibitor genistein decreases DAT activity in

striatal homogenates (162). Inhibition of dopamine uptake by genistein is both concentration-dependent and reversible. It is unlikely that changes in dopamine uptake are occurring as a result of direct actions of genistein on the DAT, as genistin, the inactive structural analog of genistein has no effect on DAT activity. Moreover, the DAT is also inhibited by an unrelated protein tyrosine kinase inhibitor, tyrphostin 23. Genistein has no effect on the endogenous GAT activity in the same preparations suggesting that tyrosine phosphorylation may only play a role in the regulation of a subset of the Na^+/Cl^- transporters.

Regulation by Protein Phosphatases

The functional regulation of proteins through phosphorylation is brought about by a balance between the activity of protein kinases and protein phosphatases. The observation that the DAT and SERT are phosphorylated in the basal state (70,147,180) suggests that in addition to the regulation of the Na^+/Cl^- transporters by protein kinases, they may be effected by the action of protein phosphatases as well.

Calyculin A (CLA), an inhibitor of both protein phosphatase (PP) 1 and PP2A increases the phosphorylation state of both the DAT and SERT over basal levels (147,180). CLA also reduces transport associated currents in *Xenopus* oocytes expressing the TAUT (96). Uptake activity of the native SERT (151), GAT (55), and TAUT (110,111) are inhibited by CLA, as is the cloned SERT expressed in COS-7 cells (151). As with PKC-mediated inhibition of the transporters, CLA decreases the V_{max} of transport (110,151), with no change in K_T (151). Furthermore, the PKC inhibitor staurosporine blocks CLA-mediated inhibition of the SERT, suggesting that either PP1 or PP2A work in concert with PKC to regulate the activity of the Na^+/Cl^- transporters.

Okadaic acid (OA), another inhibitor of PP2A, has also been examined for its effect on transporter function. Like CLA, OA increases the level of phosphorylation of

both the DAT and SERT (147,180). The OA-induced increase in DAT and SERT phosphorylation is also accompanied by a decrease in uptake activity. Moreover, OA inhibits the activity of other members of the Na^+/Cl^- transporter family (38,55,171,174). However, PP2A is unlikely to be associated with the PKC-mediated inhibition of the transporters, as the presence of OA has no effect the PMA-mediated inhibition of the DAT (186).

Little is known about the effects of other protein phosphatase inhibitors on the phosphorylation state or activity of the transporters. Microcystin increases SERT phosphorylation over basal levels, however the effects on uptake activity are unknown (147). In contrast, microcytin has no effect on the phosphorylation state of the DAT (180). Moreover, the PP2B inhibitor cyclosporin A has no effect on the phosphorylation of either the SERT or DAT (147,180), and induces only a minor increase in GAT activity (55).

Endogenous Protein Kinase Pathways and Ligands

While the use of compounds that directly stimulate protein kinases and phosphatases has facilitated the study of transporter regulation *in vitro*, the identification of endogenous signaling pathways is crucial to our understanding of how the Na^+/Cl^- transporters are regulated *in vivo*. Two recent reports have demonstrated the involvement of muscarinic acetylcholine receptors (mAChRs) in the regulation of the Na^+/Cl^- transporters. The mAChR agonists muscarine and methacholine (MCh) inhibit the activity of both the NET and GAT1 (8,13). The non-specific AChR antagonist atropine effectively blocks the mAChR-mediated inhibition of the transporters (8,13), but atropine alone has no effect on transport activity (13). The action of mAChR agonists is also blocked by the $M_1/M_3/M_5$ antagonist 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) (8,13), but not by the M_2/M_4 inhibitor himbacine (13) or the M_1 selective

inhibitor pirenzine (8), suggesting that either the M_3 or M_5 mAChR subtypes are involved in transporter regulation. The activation of mAChRs, which are coupled to phospholipase C and PKC (11,87), decreases the V_{max} of transport and the B_{max} of radioligand binding, without effecting the K_T or K_D (8,13). The mAChR-mediated inhibition of the transporters is blocked by the staurosporine, indicating that the effects of mAChR agonists are mediated by PKC (8,13). However, while the activity of the glycine transporter is inhibited by PMA, it is unaffected by activation of the mAChR (8), suggesting some specificity in the effect of muscarinic agonists. Changes in the surface expression of the transporters appears to play a role in both PMA-mediated and mAChR-mediated regulation. GAT1 is rapidly redistributed from the cell surface to an intracellular localization in the presence of either PMA or muscarine (13). Furthermore, inhibition of the GAT1 by either muscarine or PMA is blocked by botulinum toxin C1 (13,14), suggesting that regulation of the transporter is occurring through the same mechanism.

The potential role of nicotinic AChRs (nAChRs) in the regulation of transporter function is less clear. Stimulation of the nAChRs with nicotine has no effect on the activity of GAT1 (13). Moreover, the nAChR antagonist hexamethonium does not block the effects of the non-specific AChR agonist MCh on the activity of the NET (8), indicating that the nAChRs are not involved in the regulation of these two transporters. However, the nAChR agonist DMPP does inhibit the activity of the DAT in PC12 cells (69). Although inhibition of the DAT by DMPP is kinetically similar to that seen with phorbol esters (i.e. through a decrease in V_{max} with no change in K_T), and DMPP can activate PKC in PC12 cells (105), PKC inhibitors do not block the DMPP-mediated inhibition of the DAT. Therefore, regulation of the DAT through stimulation of nAChRs appears to be mediated by a PKC-independent pathway. Taken together, the results of studies on the role of AChRs in the regulation of transporter function suggest that while

the effects of PMA are quite broad, there is considerable selectivity in the response to activation of PKC-coupled receptors.

Other G-protein coupled receptors may also be involved in transporter regulation. Incubation of GAT1 expressing cells with glutamate results in an inhibition of GABA uptake (13). The effect of extracellular glutamate on GAT1 activity is attributed to the activation of metabotropic glutamate receptors (mGluRs). In addition to the inhibition of GAT1 by glutamate, the non-specific mGluR agonist *trans*-(1*S*,3*R*)-1-amino-1,3,cyclopentanedicarboxylic acid (ACDP) decreases GABA uptake. Moreover, the *trans*-ACDP mediated inhibition of GAT1 is blocked by the mGluR antagonist methyl-4-carboxyphenylglycine (MCPG) (13). Additional pharmacologic characterization indicates that Group I, and not Group III mGluRs are involved in regulating GAT1 activity. Group III mGluRs and mAChRs both couple to PKC and may inhibit GAT1 through a common pathway. Consistent with that idea, the combined effects of subsaturating concentrations of mAChR agonists and mGluR agonists are not synergistic (13). Moreover, neither mAChR agonists nor mGluR agonists potentiate the PMA-mediated inhibition of GAT1 (8,13).

Extracellular serotonin also inhibits GABA uptake in cultures of hippocampal neurons (13). The type 2 serotonin receptor is at least partially responsible for the regulation of GAT1 activity, as the type 2-specific serotonin receptor agonist α-methyl-serotonin also inhibits GABA uptake (13). However, the participation of other serotonin subtypes in the regulation of the GAT1 has not been examined. The type 2 serotonin receptor couples to PKC, although the role of PKC in the effect of serotonin receptor activation on transporter activity has not been fully characterized.

Although extracellular dopamine has no effect on the activity of the GAT1 (13), there is evidence that activation of the D_2 dopamine receptor modulates the activity of the DAT. The D_2-specific agonist quinpirole causes in an increase in dopamine transport

that is blocked by D_2 antagonists (100). Local application of the D_2 antagonist raclopride also inhibits dopamine clearance *in vivo* in the medial prefrontal cortex, dorsal striatum, and nucleus accumbens of rats (29). Furthermore, D_2 receptor knockout mice exhibit decreased dopamine clearance in the striatum (43). Taken together, these results suggest that in addition to its role in regulating dopamine release (reviewed in 88), the presynaptic autoreceptor D_2 is involved in regulating the activity of the DAT. Studies on the activation of the D_2 receptor provide the first evidence that the activity of a Na^+/Cl^- transporter is stimulated in response to activation of a G-protein coupled receptor, however, the kinase pathway involved has not been identified.

G-protein coupled receptors that activate the NO/cGMP signaling pathway are also involved in regulation of the Na^+/Cl^- transporters. Activation of the adenosine A3 receptor by the agonists 5'-*N*-carboxamidoadenosine (NECA) or N6-cyclopentyladenosine stimulates SERT activity, an effect that is mimicked by NO generators and 8-br-cGMP (106). Furthermore, the adenosine A3 receptor-mediated increase in serotonin uptake is blocked not only by receptor antagonists, but by inhibitors of nitric oxide synthase (NOS) and PKG as well. The activity of the platelet SERT is also increased by incubation with histamine, through stimulation of an H2-type histamine receptor (89). Histamine-mediated upregulation of the SERT is inhibited by both H2 receptor antagonists, and NOS inhibitors, suggesting that the H2-like receptor is coupled to the NO/PKG pathway. The involvement of PKG in SERT regulation is further demonstrated by the ability of the cGMP-inducing agent nitroprusside to increase SERT activity in isolated human platelets (89).

The Na^+/Cl^- transporters are also subject to regulation by substrates and inhibitors. PMA-mediated phosphorylation of the SERT expressed in HEK-293 cells is diminished by the presence of serotonin or the transported inhibitors amphetamine and fenfluramine (144). Non-transported inhibitors, such as cocaine and paroxetine block the

serotonin-mediated decrease in phosphorylation, but alone have no effect on PMA-mediated SERT phosphorylation. The presence of serotonin completely reverses the PMA-mediated translocation of the SERT from the cell surface to an intracellular localization (144). Since internalization of the SERT is responsible for the decrease in uptake activity mediated by PMA (138), the presence of extracellular substrate appears to completely ameliorate inhibition of SERT by PKC. Phosphorylation of the DAT also appears to be reduced by the presence of extracellular dopamine, although this finding has not been thoroughly investigated (182).

The activity of both the native and the cloned GAT1 are directly modulated by the presence of substrates and inhibitors. Incubation of GAT1 expressing cells with GABA or with the transported inhibitors nipecotic acid and ACHC induces an increase in GAT1 uptake that is blocked by non-transported inhibitors (19). The GAT1-specific inhibitor SKF89976A not only inhibits the substrate-induced upregulation of GAT1, but also acts to downregulate GAT1 activity. The substrate and inhibitor induced changes in GAT1 activity are accompanied by changes in cell surface expression. While overall protein levels remain unchanged, GAT1 protein at the cell surface increases following incubation with GABA and decreases following incubation with SKF89976A.

Substrate-mediated changes in the uptake activity and phosphorylation of the transporters are not due to receptor activation. The serotonin-mediated decrease in PKC phosphorylation of the SERT is not blocked by the presence of serotonin receptor antagonists (144). Likewise, the GABA-mediated increase in uptake activity is unaffected by the presence of GABA receptor inhibitors (19). Therefore, substrate-mediated alterations in transporter activity and phosphorylation appear to be the result of direct effects on the transporters. Conformational changes in the transporters induced by the translocation of substrate have been proposed to underlie the alterations in uptake activity and phosphorylation seen in the presence of substrate. This is supported by the

observation that while Na^+ and Cl^- are not necessary for the PMA-mediated phosphorylation of the SERT, they are required for the serotonin-mediated reduction in phosphorylation (144). The extracellular domains of transporters are believed to be involved in the responses to the presence of substrate, as intracellular substrate concentrations have no effect on the substrate-mediated alterations in phosphorylation or uptake activity (19,144). Changes in transporter conformation induced by substrates and inhibitors may alter interactions with cellular kinases or accessory proteins that are involved in transporter regulation.

Chronic Regulation of the Na^+/Cl^--dependent Neurotransmitter Transporters

In addition to acute regulation arising from protein kinase activation, the Na^+/Cl^- transporters are subject to long-term regulatory mechanisms. While the role of PKA remains controversial, it does not appear to play an important role in the acute regulation of the transporters. However, there is significant amount of evidence that PKA is involved in the chronic regulation of transport activity. Long term exposure to cholera toxin (CTX) (16-24 hours) increases the activity of the native SERT expressed in JAR cells (37,80,145), and the native TAUT expressed in HPRE cells (46,108). While CTX induces a rapid rise in cAMP levels (37,80), there is no increase in transport activity for at least 4 hours, and maximal uptake activity is only seen 16-24 hours after chronic exposure to CTX (37,46,80). Chronic exposure to the PKA activators forskolin and 3-isobutyl-1-methylxanthine (IBMX) produces the same effect as CTX (37,80), as do the cAMP analogs dbcAMP and 8-br-cAMP (37,46,108). Moreover, chronic modulation of the transporters mediated by CTX is blocked by PKA inhibitors (37,108). The increased activity of the transporters results from an increase in both the V_{max} of transport (37,46) and B_{max} of radioligand binding (145), while the affinity for substrates and inhibitors is unaffected (37,46,145). Upregulation of transport activity is also abolished by the

presence of transcription and protein synthesis inhibitors (46,145), indicating that unlike acute regulation of the transporters, chronic regulation results from an increase in protein synthesis. While protein levels were not examined, the marked increase in steady state mRNA levels that accompanies chronic upregulation of the transporters implies that there is an accompanying increase in transporter protein (46,145).

Although the results of long-term exposure to CTX are consistent for the SERT in JAR cells and the TAUT in HRPE cells, chronic regulation of the transporters by PKA may be highly specific to both the cell type and the transporter expressed. JAR cells express both the SERT and TAUT, and although the SERT is upregulated by chronic exposure to CTX, there is no effect on either taurine uptake activity (37) or TAUT mRNA levels (145). Furthermore, the activity of the native SERT in PC12 cells is inhibited by CTX, under the same conditions that stimulate SERT activity in JAR cells (80). When cultured neurons are incubated with forskolin for 24 hours there is a dramatic decrease in both GABA uptake activity and GAT1 mRNA (53), the opposite of what is seen with the SERT and TAUT. Furthermore, there is no change in the levels of GLYT1 mRNA in the same cells (53).

The Na^+/Cl^- transporters are also subject to chronic regulation by kinases other than PKA. The properties of interleukin-1β (IL-1β)-mediated upregulation of the SERT are similar to those seen when PKA activity is stimulated by CTX. Exposure of JAR cells to IL-1β for 16 hours significantly increases serotonin uptake (143). The onset of the IL-1β-mediated increase in SERT activity is delayed for several hours and involves an increase in V_{max} and B_{max} with no change in K_T or K_D. While the identity of the kinase involved remains unknown, the effects of IL-1β on the SERT appear to be independent of PKA activation. There is no evidence of an increase in cyclic nucleotide levels following incubation with IL-1β, and the effects of CTX and IL-1β are synergistic, suggesting that IL-1β and CTX are acting through separate pathways. Although the activation of PKC

plays an important role in the acute regulation of the transporters, it does not appear to be involved in chronic regulation. Long-term (16 hour) incubation of JAR cells with PMA has no effect on SERT activity (143).

Tyrosine phosphorylation may be involved in chronic regulation of the transporters as well as acute regulation. The SERT is upregulated following incubation of JAR cells with the neuroprotective agent aurintricarboxylic acid (ATA) (78). ATA-mediated stimulation of SERT activity is again characterized by delayed onset, an increase in V_{max} and B_{max} unaccompanied by a change in affinity, and increased mRNA levels. ATA stimulates multiple pathways involving tyrosine phosphorylation (122) and may protect neurons from cell death by mediating cellular functions normally associated with growth factors. This hypothesis is supported by the observation that epidermal growth factor (EGF) is able to reproduce the effects of ATA on SERT function and mRNA levels (78). Furthermore, the tyrosine kinase inhibitor genistein blocks both ATA-mediated and EGF-mediated upregulation of the SERT, suggesting that ATA is acting through a PTK.

In addition to the effects of protein kinase activators, protein kinase inhibitors also mediate the chronic regulation of SERT activity. Both the serine/threonine kinase inhibitor staurosporine and the PTK inhibitor herbimycin A modulate SERT activity in JAR cells (136,143). Surprisingly, inhibition of protein kinases has the same effect on the SERT as does activation of protein kinases. Long-term incubation of JAR cells with either staurosporine or herbimycin A results in a delayed increase in SERT activity which arises from an increase in V_{max} and B_{max} with no change in affinity and is accompanied by an increase in SERT mRMA levels. The explanation for the seemingly discordant actions of kinase activation and kinase inhibition may lie in the complexity of kinase interactions. While the PTK inhibitor genistein itself has no effect on SERT activity, genistein is able to block the herbimycin A-mediated stimulation of the SERT (136). Furthermore, the phosphotyrosine content of several proteins is increased during incubation of JAR cells

with herbimycin A. These results suggest that a cascade of PTKs is involved in chronic SERT regulation. The tyrosine phosphorylation mediated by herbimycin A may reflect the activation of downstream PTK that is directly involved with the modulation of the SERT. Staurosporine may be stimulating SERT activity through a similar mechanism. Staurosporine has been shown to induce the expression of the EGF receptor in PC12 cells (142). While there is no direct evidence for an increase in EGF receptors in staurosporine-treated JAR cells, stimulation of the EGF receptor is able to upregulate SERT activity (78) and may be responsible for the staurosporine-mediated regulation of the SERT.

As with the acute regulation of the transporters, incubation with substrate or inhibitor can effect the chronic regulation of the carriers. Incubation of PC12 cells with the NET inhibitor desipramine for 3 days results in an inhibition of the endogenous NET activity (188). While the NET mRNA and protein levels were not examined, the resulting reduction in the B_{max} of nisoxetine binding with no change in affinity suggests the number of carriers is diminished. Incubation of the cells with the structurally unrelated inhibitor nisoxetine, or with norepinephrine also inhibits NET activity. In contrast, the SERT-selective inhibitor citalopram has no effect on the activity of the NET. The activity of the native TAUT is also modulated by prolonged incubation of JAR, MDCK, or CaCo-2 cells with taurine. After 24 hours of exposure to extracellular taurine, both taurine uptake and TAUT mRNA levels are significantly reduced in all 3 cell lines (73,74,154). Incubation of the cells with other TAUT substrates has a similar effect, while TAUT activity is unaltered by the presence of related non-substrates such as GABA and betaine (73,154). High extracellular levels of substrate might be expected to stimulate rather than inhibit transporter activity. However, as these cell lines all accumulate taurine very efficiently, high intracellular concentrations associated with the prolonged presence of extracellular substrate may trigger downregulation of the transporter.

In summary, due to the importance of the reuptake systems in modulating CNS function, the activity of neurotransmitter transporters is believed to be tightly controlled. The Na^+- and Cl^--dependent neurotransmitter transporters are subject to the regulatory influence of many protein kinases and phosphatases. The diversity of effects mediated by stimulation of signaling pathways reflects the complex role that the network of kinases and phosphatases play in regulating the activity of proteins and cellular processes, including the activity of neurotransmitter transporters. Indeed, many regulatory molecules may act in concert to fine tune transporter function. Furthermore, the actions of a single second messenger may effect multiple kinase cascades. The variety of responses also underscores the difficulty encountered when trying to understand how protein kinases regulate the activity of the transporters.

Regulation of Three Members of the Na^+- and Cl^--dependent Neurotransmitter Transporter Family by Protein Kinase C.

Gwynn M. Daniels‡¶ and Susan G. Amara‡§¶

From the ‡Department of Cell and Developmental Biology, §Howard Hughes Medical Institute, and the ¶Vollum Institute, Oregon Health Science University, Portland, Oregon 97201.

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SUMMARY

The dopamine transporter, β-alanine-sensitive γ-aminobutyric acid transporter, and norepinephrine transporter are all members of the Na^+- and Cl^--dependent family of neurotransmitter transporters. When MDCK cells stably expressing the cloned dopamine, γ-aminobutyric acid, or norepinephrine transporters were treated with the phorbol ester phorbol 12-myristate 13-acetate, a potent activator of protein kinase C, uptake of radiolabeled substrate was decreased by 40-60%. Phorbol ester-mediated inhibition of the transporters was both time- and concentration-dependent and appeared to be irreversible. The observed decrease in uptake activity was specific to protein kinase C activation, as the inactive phorbol ester 4-α-phorbol had no effect on transporter function, and downregulation of the carriers was blocked by the protein kinase C inhibitor staurosporine. The reduction in the activity of the carriers upon stimulation with phorbol esters resulted from a decrease in V_{max}, while the apparent affinity of the transporters for substrate remained unchanged. Treatment with phorbol esters significantly reduced the number of transporter molecules found at the cell surface. Examination by indirect immunofluorescence showed a dramatic shift in the localization of the carriers from the plasma membrane to a subcellular compartment following incubation with phorbol esters. These results indicate that redistribution of the carriers underlies the downregulation of Na^+/Cl^- transporters by protein kinase C.

INTRODUCTION

Neurotransmitter transporters play an essential role in the modulation of synaptic activity. Reuptake of neurotransmitter into the presynaptic neuron or surrounding glia results in the termination of synaptic transmission, and provides a mechanism for the recycling of neurotransmitter molecules. Because the activity of the neurotransmitter transporters plays an important role in determining synaptic strength, the functional regulation of the carriers is of considerable interest.

Several members of the Na^+/Cl^--dependent neurotransmitter transporter family have been cloned, including transporters specific for the reuptake of dopamine (50,79,160), GABA (35,58,94,97), norepinephrine (125), serotonin (22,67), taurine (166,176) and betaine (184). The deduced amino acid sequences of the cloned transporters reveals a high degree of sequence identity (60-90%), as well as the presence of several common structural features. The dopamine transporter (DAT), β-alanine sensitive GABA transporter (GAT3), and norepinephrine transporter (NET) all contain multiple consensus sites for phosphorylation by protein kinases, suggesting that phosphorylation may be involved in regulating transporter activity.

Recent evidence suggests that the activity of the Na^+/Cl^--dependent neurotransmitter transporters is regulated by protein kinase C (PKC). When the neuronal GABA transporter GAT1 is expressed in *Xenopus* oocytes, uptake activity is markedly increased in response to phorbol esters (39). This PKC-mediated increase in GAT1 activity results from recruitment of transporters to the cell surface, in a manner similar to that of the insulin-dependent glucose transporters (41,169). Furthermore, the translocation of GAT1 to the plasma membrane in response to PMA is dependent on its association with the trafficking-related proteins syntaxin and SNAP-25 (139). While the

regulation of GAT1 by PKC has been studied in some detail, the effect of phorbol esters on GAT3, another neuronally expressed GABA transporter subtype, has not been examined.

The function of the monoamine transporters, which includes the serotonin transporter (SERT) as well as the DAT and the NET, are of particular interest because they are the site of action of both drugs of abuse and therapeutic antidepressants. In contrast to GAT1, the activity of the DAT is decreased by phorbol esters both in synaptosomes and in heterologous expression systems (38,70,81,186,189). Activation of PKC also inhibited the accumulation of serotonin into cells that express the SERT either endogenously (6,114,151) or exogenously (138,151). Cell surface biotinylation suggested that PMA-mediated inhibition of SERT activity is due to a loss of transporter protein at the plasma membrane (138). Moreover, a decrease in both inhibitor binding and membrane capacitance accompanies the PKC-mediated inhibition of uptake activity in *Xenopus* oocytes expressing the DAT, again suggesting that redistribution underlies downregulation of the transporter (189). The DAT also shows increased phosphorylation in response to application of PMA (70), although a direct correlation between phosphorylation and an inhibition of uptake activity has not been demonstrated. While downregulation of both the DAT and the SERT by PKC is believed to result from a loss of transporter molecules at the cell surface, the subcellular localization of these molecules, in either the presence or absence of PMA, has not been examined. In addition, the role of protein kinases in the regulation of the NET has yet to be determined.

In the present study we have examined the effect of PKC stimulation on three members of the Na^+/Cl^--dependent neurotransmitter transporter family. Using MDCK cells stably expressing rat DAT, rat GAT3, or human NET we find that the activation of PKC by phorbol esters results in a decrease of both transport activity and cell surface

expression. We also show that the regulation of the transporters is the result of a rapid redistribution of the carriers from the plasma membrane to a population of intracellular vesicles.

EXPERIMENTAL PROCEDURES

Materials - LIPOFECTAMINE™ reagent, Dulbecco's Modified Eagle's Meduim (DMEM), fetal calf serum, and antibiotics were purchased from GIBCO/BRL. Tritiated substrates were acquired from New England Nuclear. Forskolin, 3-isobutyl-1-methylxanyhine (IBMX), phorbol 12-myristate 13-acetate and 4-α-phorbol were from Calbiochem. NHS-SS-Biotin and UltraLink Immobilized Neutravidin were obtained from Pierce.

Plasmid Construction and Generation of Stable Cell Lines - The coding sequences of the rat DAT, rat GAT3, and human NET were subcloned into the *Xho1* and *Xba1* sites of the expression plasmid pCMV5 (7), to generate constructs (termed pCMV5-DAT, etc.) for stable transfection into MDCK cells.

MDCK cells (ATCC) were grown to ~80% confluence in 35 mm tissue culture dishes. Cells were exposed to a solution of 1.5 μg plasmid DNA (pCMV5-DAT, pCMV5-GAT3, pCMV5-NET, or pCMV5), 0.15 μg pRSV*neo*, and 40 μg LIPOFECTAMINE™ reagent in serum-free DMEM for 5 h, after which the DNA/cationic lipid solution was replaced with DMEM supplemented with 10% FCS, 10 U/ml penicillin, and 10 μg/ml streptomycin. 72 h post-transfection the cells were plated into DMEM supplemented with 10% FCS and 0.5 mg/ml G418, and incubated at 37°C in 5% CO2 for 2 weeks with frequent changes of media. Resistant colonies were selected using cloning rings, and screened for transporter expression by uptake of [^{3}H]dopamine, [^{3}H]GABA, or [^{3}H]norepinephrine. Stable lines were maintained in DMEM supplemented with 10% FCS and 0.5 mg/ml G418.

Uptake Assay - MDCK-DAT, MDCK-GAT3, MDCK-NET, and MDCK-CMV cells were grown to confluence (2-3 days after plating). The cells were washed twice in 37°C Krebs-Ringers-Hepes (KRH) buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl2, 1.2

mM MgSO4, 1.2 mM KH2PO4, 10 mM HEPES, pH 7.4), and then incubated in KRH buffer for 10 min at 37°C. Uptake was initiated by the addition of varying concentrations of dopamine, GABA, or norepinephrine and was allowed to proceed for 10 min at 37°C. The concentration of [^{3}H]dopamine, [^{3}H]GABA, or [^{3}H]norepinephrine for all experiments was 50 nM, 25 nM, and 20 nM respectively. Uptake was terminated by rapidly washing the cells 3 times in ice cold KRH buffer. The cells were solubilized in 0.1 N NaOH, 0.1% SDS and radioactivity was assessed by liquid scintillation counting. Non-specific counts were considered to be those accumulated by MDCK-CMV cells in parallel experiments. The results were analyzed using Excel and Kaleidagraph software.

Immunofluorescence - MDCK-DAT, MDCK-GAT3, and MDCK-NET cells were grown to near confluence on glass coverslips. The coverslips were washed twice in PBS, and fixed for 20 min in freshly prepared 4% paraformaldehyde. After fixation the coverslips were washed 3 times in PBS and then permeabilized and blocked for 20 min at room temperature in PBS containing 1% BSA, 2% normal horse serum, and 0.5% Triton X-100. Following the blocking and permeabilization, the coverslips were washed 3 times in PBS containing 1% BSA and 2% normal horse serum (IF buffer), and incubated for 2-3 h at room temperature with primary antibody diluted in IF buffer. Following incubation with primary antibody the coverslips were washed 3 times with IF buffer and incubated with fluorescent secondary antibody diluted in IF buffer for 1 h. After incubation with secondary antibody the coverslips were washed 3 times with IF buffer, once with PBS, and mounted on glass slides with *p*-phenylenediamine in 50% glycerol. The slides were imaged on a BIO-RAD krypton/argon laser scanning confocal microscope.

Cell Surface Biotinylation and Western Blotting - Cell surface biotinylation performed essentially as described (see Appendix). MDCK-DAT, MDCK-GAT3, and MDCK-NET cells were grown to near confluence in 6-well plates. Following treatment with PMA or vehicle alone, the cells were washed in ice cold PBS, and incubated at 4° with

biotinylation buffer (150 mM NaCl, 2 mM $CaCl_2$, 10 mM triethanolamine, pH 7.5) containing 2 mg/ml NHS-SS-biotin (Pierce). The biotinylation reaction was quenched by washing the cells extensively in quench buffer (100 mM glycine in PBS). The cells were scraped into 1 ml lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5, 1% Triton X-100) and the proteins were extracted for 1 hr on ice. The cell extracts were cleared, and the supernatant was incubated with UltraLink Immobilized Neutravidin (Pierce) overnight at 4°C. The Neutravidin beads were washed 3 times in lysis buffer, twice in high salt wash buffer (500 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5, 0.1% Triton X-100), once in 50 mM Tris, pH 7.5, and incubated for 10 min at room temperature in 2X SDS sample buffer followed by a 30 min incubation at 37°C.

Biotinylated proteins or cell extracts were separated on 8% SDS-polyacrylamide gels, transferred to Immobilon P membrane (Millipore), and probed with α-DAT (1:500) or α-GAT3 (1:500) antisera, or affinity purified α-NET antibody (1 μg/ml). The blots were then incubated with peroxidase conjugated secondary antibody and developed with Enhanced Chemiluninescence reagent (NEN).

Antibodies - Polyclonal antisera VO436 was generated against the amino-terminal 57 amino acids of rDAT. Polyclonal antisera VO1810, was raised against the amino-terminal 54 amino acids of rGAT3. Polyclonal α-NET antibody was affinity purified from antisera VO1210 as previously described (119). Fluorescein-conjugated donkey α-rabbit IgG, and HRP-conjugated donkey α-rabbit IgG were obtained from Jackson.

RESULTS

Stable transfection and characterization of MDCK cells - The study of endogenously expressed neurotransmitter transporters is complicated by a number of factors, including difficulty in obtaining sufficient material for assay, and the presence of other proteins that may indirectly effect transport. For example, the presence of vesicular reuptake systems may increase the apparent activity of plasma membrane transporters by altering the concentration gradient of substrate. Therefore, we have examined the regulation of function of the Na^+/Cl^--dependent neurotransmitter transporter family in an exogenous mammalian expression system. MDCK cells were chosen because they previously have proven useful as a model system for the cellular trafficking of neuronally expressed proteins such as Thy-1 (135) and N-CAM 180 (134). Most membrane proteins that are localized to the somatodendritic domain of the plasma membrane in neurons are targeted to the basolateral surface of MDCK cells (reviewed in 40). Conversely, those proteins that are axonally restricted in neurons are generally targeted to the apical surface of MDCK cells. The results of these studies suggest that epithelial cells may express cellular components involved in protein trafficking that are similar to those expressed in neurons.

DAT, GAT3, and NET cDNAs were subcloned into the vector pCMV5, under the control of the cytomegalovirus promoter, to produce pCMV5-DAT, pCMV5-GAT3, and pCMV5-NET. The resulting plasmids were used to transfect MDCK cells, generating cell lines that stably express DAT, GAT3, and NET, and were designated MDCK-DAT, MDCK-GAT3, and MDCK-NET, respectively. MDCK cells were also stably transfected with pCMV5 vector alone, and were designated MDCK-CMV.

The specific uptake of radiolabeled substrate was examined for each cell line. The accumulation of tritiated substrate by these cell lines exceeded background uptake in

MDCK cells harboring the pCMV5 vector alone by approximately 150-fold for DAT, 15-fold for GAT3, and 100-fold for NET (Fig. 1A). MDCK cells exhibit low endogenous GABA uptake activity, presumably through the endogenously expressed betaine transporter, which is also able to accumulate GABA at high affinity (184). Uptake activity for all clones was substrate specific, and was linear for at least 15 min at 37°C (data not shown).

To examine the presence of carrier protein in MDCK-DAT, MDCK-GAT3, and MDCK-NET cells, whole cell lysates from transfected cells were probed with antisera specific to rDAT, rGAT3, and hNET, respectively (Fig. 1B). The MDCK-DAT blot was probed with antisera VO436, that recognizes a broad band centered at approximately 89 kilodaltons (kD) in the DAT transfected cells that is absent from the CMV5 transfected cells. These broad bands are typical of transporter molecules, and represent multiple glycosylated forms of the protein (119). This antisera also recognizes three non-specific bands of approximately 97, 81, and 61.5 kD in both MDCK-DAT and MDCK-CMV5 cells. These non-specific bands were present even when the antisera was preincubated with up to 20 μg of MDCK-CMV5 cell lysate (data not shown). The MDCK-GAT3 blot was probed with antisera VO1810, that recognizes a single band centered at approximately 93 kD in MDCK-GAT3 cells that was not present in MDCK-CMV5 cells, as well as a minor band which was present in both cell lines. The MDCK-NET blot was probed with affinity purified α-NET antibody that recognized a single band of approximately 84 kD present only in the NET expressing cells. The molecular weights suggested by these data are consistent with the predicted molecular weights for mature DAT, GAT3, and NET.

Activation of Protein Kinase C Inhibits Substrate Translocation - Activators of protein kinases were examined for their effect on the function of DAT, GAT3, and NET. Phorbol esters directly stimulate PKC by substituting for the endogenous activator

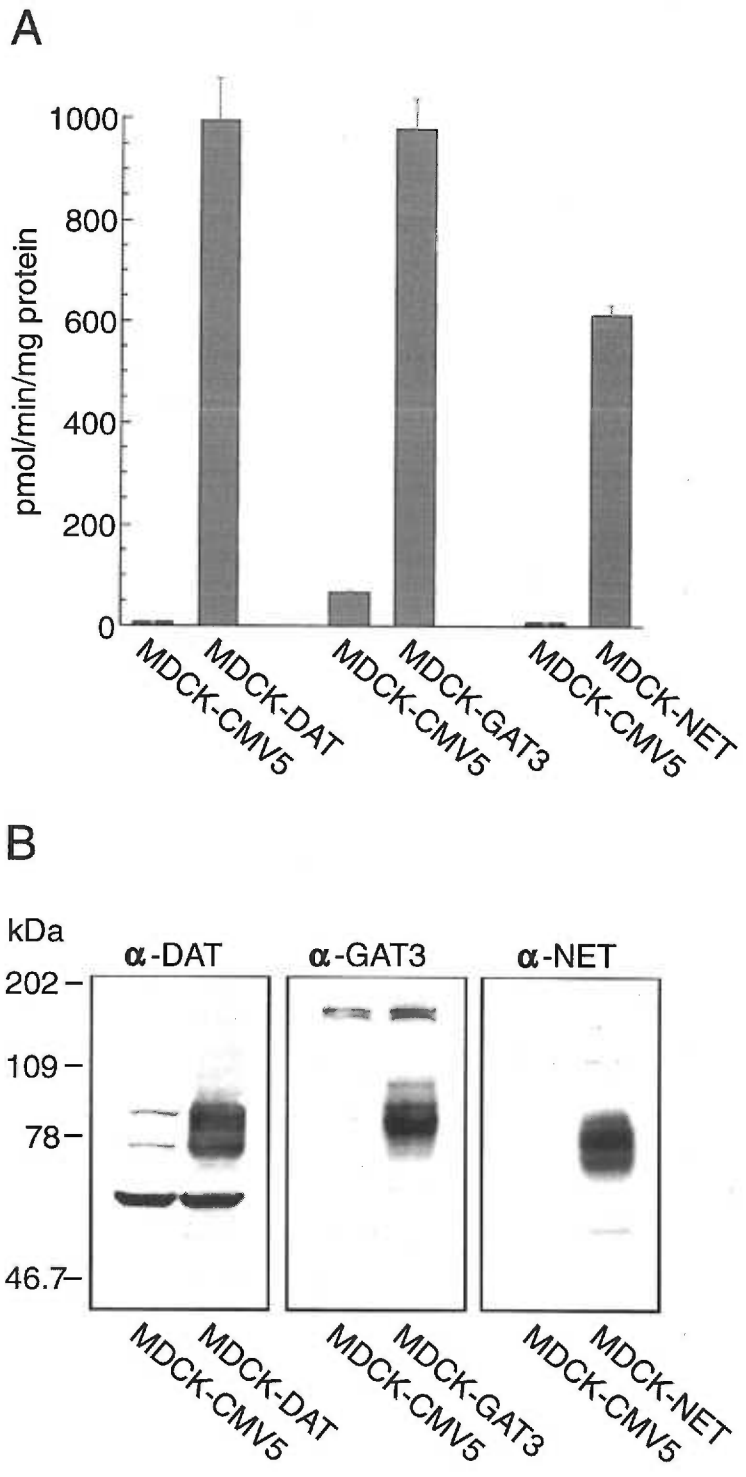

A
1000
800
600
400
200
0
pmol/min/mg protein
MDCK-CMV5
MDCK-DAT
MDCK-CMV5
MDCK-GAT3
MDCK-CMV5
MDCK-NET
B
kDa
α-DAT
α-GAT3
α-NET
202
109
78
46.7
MDCK-CMV5
MDCK-DAT
MDCK-CMV5
MDCK-GAT3
MDCK-CMV5
MDCK-NET

Figure 1. **Characterization of MDCK-DAT, MDCK-GAT3, and MDCK-NET cells.** *A*, Uptake of radiolabeled substrate in MDCK cells expressing DAT, GAT3, or NET compared to uptake in cells expressing CMV5 vector alone. Cells were exposed to either [^{3}H]-dopamine, [^{3}H]-GABA, or [^{3}H]-norepinephrine for 10 min at 37°C. Uptake is expressed as pmol of substrate/min/mg of total protein ± s.e.m. Shown is a representative experiment of 3 separate experiments performed in triplicate. *B*, Immunoblots of total cell extracts (20 μg) from MDCK-DAT, MDCK-GAT3, MDCK-NET, and MDCK-CMV5 cells. Blots were probed with α-DAT or α-GAT3 antisera, or with affinity purified α-NET antibody. Prestained molecular weight standards were run in parallel.

diacylglycerol (31). When MDCK-DAT, MDCK-GAT3 and MDCK-NET cells were treated with the phorbol ester PMA, uptake activity of all three transporters was reduced by approximately 40-60% (Fig. 2). Inhibition of the transporters was specific to the activation of PKC, as incubation with the inactive isomer of PMA, 4-α-phorbol, had no effect on the activity of the transporters. In addition, while the PKC inhibitor staurosporine itself had no effect on the activity of the transporters, preincubation of the cells with staurosporine prevented inhibition by PMA. Furthermore, when the endogenous PKC activity was depleted by exposing the cells to 100 nM PMA overnight (116), subsequent treatment with additional PMA had no effect on uptake activity when compared to vehicle treated controls (data not shown).

Signal transduction through the cyclic AMP (cAMP)-dependent Protein Kinase (PKA) pathway is stimulated by an increase in the intracellular concentration of cAMP. The activation of adenylyl cyclase (AC) or the inhibition of phosphodiesterase (PDE) both result in increased levels of cAMP, and thereby activate PKA. In contrast to the inhibition of the transporters seen following incubation with PMA, stimulation of PKA through activation of AC with forskolin, or inhibition of PDE with IBMX had no effect on the amount of radiolabeled substrate accumulation for DAT, GAT3, or NET (Fig. 2). Taken together, these data suggest that the activity of the Na^+/Cl^--dependent transporters is specifically regulated by the activation of PKC.

PMA-mediated Inhibition of Transport Activity is Time- and Concentration-dependent - To determine the dose-response relationship for PMA-induced inhibition of uptake activity, MDCK-DAT, MDCK-GAT3 and MDCK-NET cells were exposed for 1 h to concentrations of PMA ranging from 10 pM to 1 μM (Fig. 3A). All three transporters showed some inhibition of uptake activity (2.8-10.2% less than vehicle treated control) in response to 10 pM PMA. Transport inhibition increased with increasing concentrations of PMA, and was maximal with approximately 10 nM PMA.

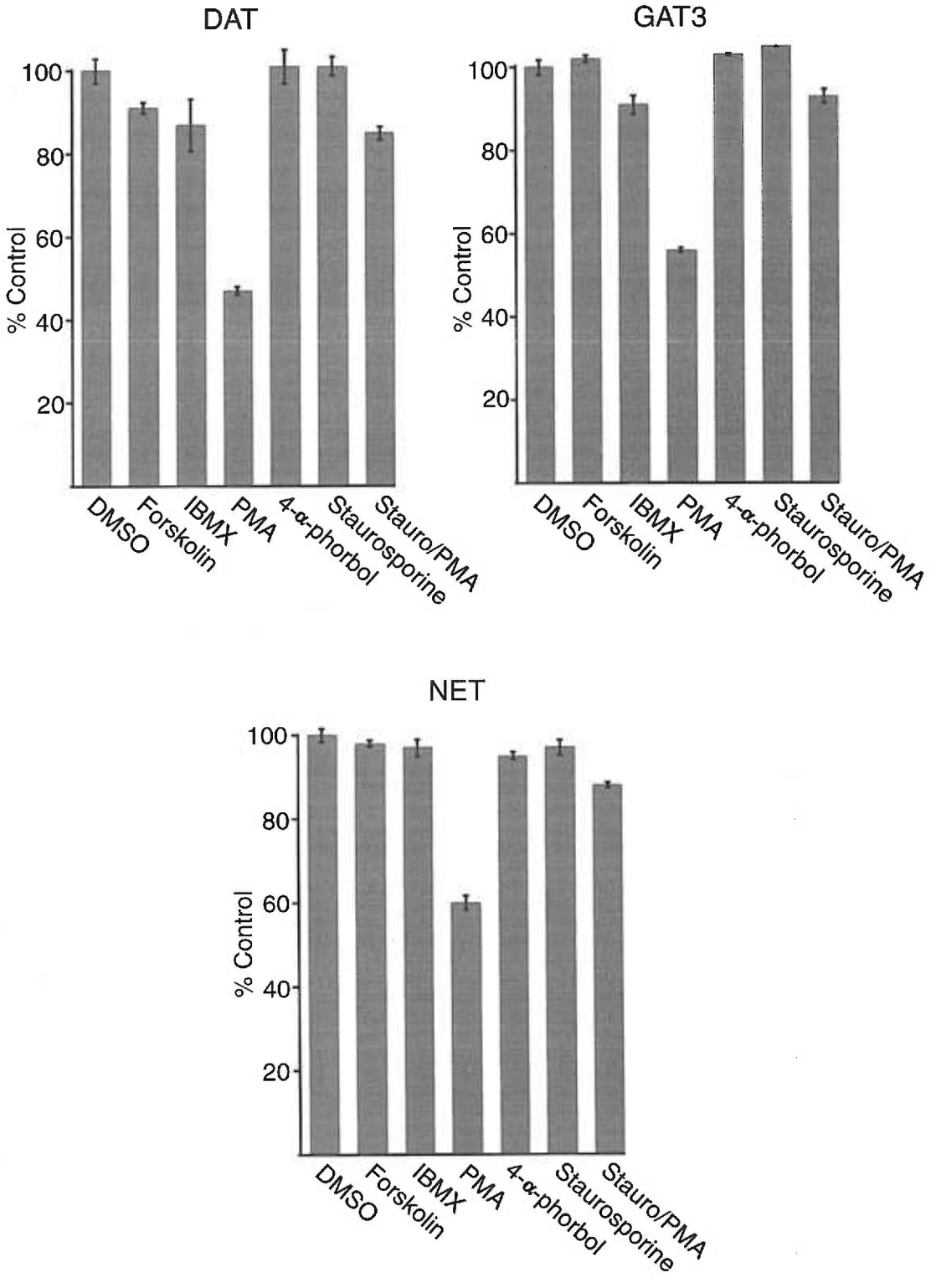

DAT
GAT3
NET
% Control
100
80
60
40
20
DMSO
Forskolin
IBMX
PMA
4-α-phorbol
Staurosporine
Stauro/PMA

Figure 2. **The uptake activity of DAT, GAT3, and NET is decreased in response to PMA.** MDCK-DAT, MDCK-GAT3, and MDCK-NET cells were treated with DMSO vehicle, 10 μM forskolin, 100 μM IBMX, 100 nM PMA, or 100 nM 4-α-phorbol for one h prior to uptake assay. Additional cells were pretreated for 15 min with 100 nM staurosporine prior to the addition of DMSO or 100 nM PMA, and incubation was continued for one h. Values shown are the percent of uptake activity compared to untreated control cells ± s.e.m. Data shown are representative of 5 separate experiments performed in triplicate.

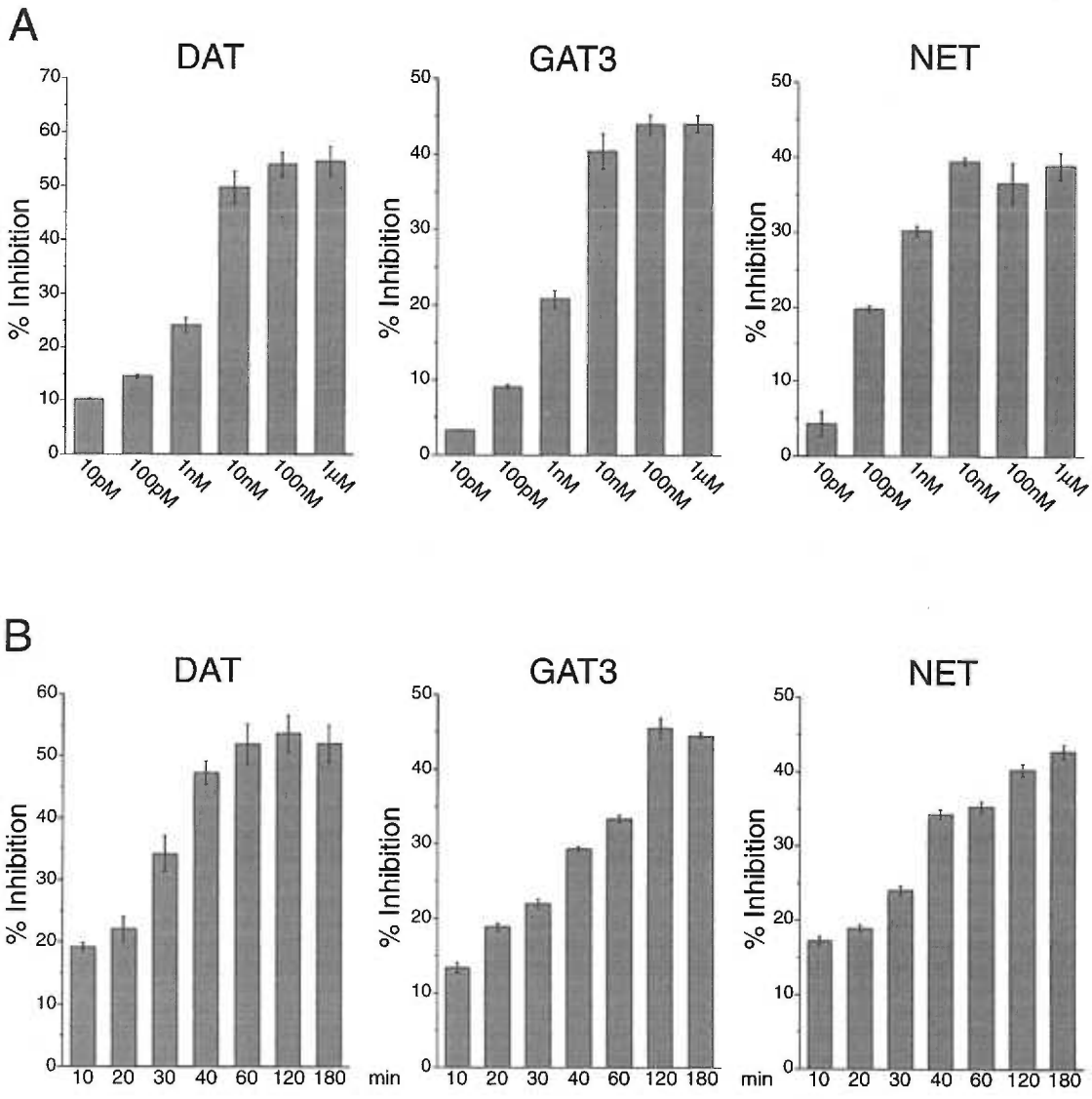

A
DAT
GAT3
NET
% Inhibition
10pM
100pM
1nM
10nM
100nM
1μM
B
DAT
GAT3
NET
% Inhibition
10 20 30 40 60 120 180
min

Figure 3. **Time- and concentration-dependence of PMA-mediated inhibition of uptake activity.** *A*, MDCK-DAT, MDCK-GAT3, and MDCK-NET cells were treated with increasing concentrations of PMA for 1 h prior to uptake assay. *B*, MDCK-DAT, MDCK-GAT3, and MDCK-NET cells were treated with 100 nM PMA for periods of time ranging from 10 to 180 min prior to uptake assay. Values shown are the percent of inhibition of uptake activity compared to vehicle treated control cells ± s.e.m., and are representative of 3 independent experiments performed in triplicate.

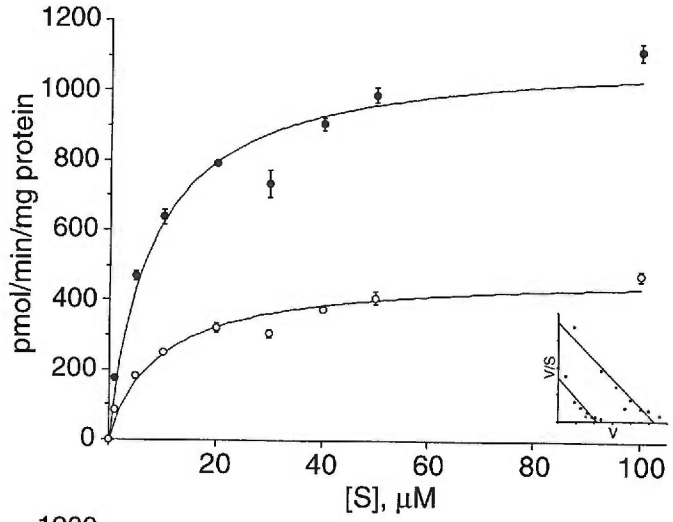
To examine the time course of transport inhibition following the PMA-mediated activation of PKC, MDCK-DAT, MDCK-GAT3, and MDCK-NET cells were treated with 100 nM PMA for periods ranging from 10 min to 3 h (Fig. 3B). Uptake activity decreased rapidly, and was inhibited by approximately 20% in all three cell lines within 10 min of exposure to PMA. Inhibition of transport activity increased over time, and was near maximum after 1 h exposure to PMA. Continued exposure to PMA for up to 3 h did not result in a significant increase in the inhibition of transporter activity.

Based on these results, MDCK-DAT, MDCK-GAT3, and MDCK-NET cells were incubated with 100 nM PMA for 1 h for all additional experiments.

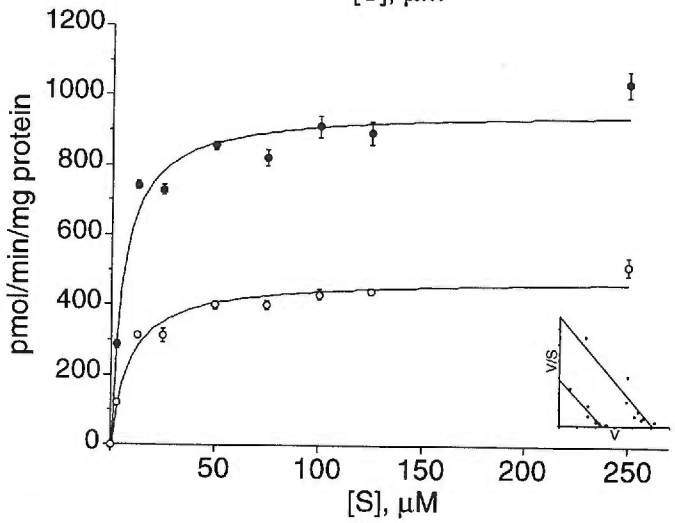
PMA Decreases Maximal Velocity, but Does Not Change the Apparent Affinity of the Transporters for Substrate- A decrease in the accumulation of substrate by the transporters could be accounted for by either a decrease in the affinity for substrates, or from a decrease in transport velocity. Therefore, the kinetics of transport activity were examined for DAT, GAT3, and NET. Transport velocity (in pmol/min/mg of protein), as a function of substrate concentration, was fitted by least-squares to the Michaelis-Menton equation (Fig. 4). All three of the transporters exhibited uptake activity that was both dose-dependent and saturable when incubated with either 100 nM PMA or an equal volume (0.1%) of DMSO vehicle alone. Values for the transport constant (K_T) and maximal velocity (V_{max}) were determined using the Eadie-Hofstee transformations (insets Fig. 4). The V_{max} of all three of the transporters was decreased in the presence of PMA (Table 1). However, the affinity of the carriers for substrate was not significantly altered.

Transporter Protein at the Cell Surface is Decreased in Response to PMA - The observed decrease in V_{max} with no accompanying change in K_T suggests that inhibition of uptake activity results from either a decrease in the turnover rate of the transporters or a loss of transporter protein from the cell surface. Alteration of cell surface expression

DAT


1200
1000
800
600
400
200
0
pmol/min/mg protein
20
40
60
80
100
[S], µM
V/S
V

GAT3


1200
1000
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0
pmol/min/mg protein
50
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150
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[S], µM
V/S
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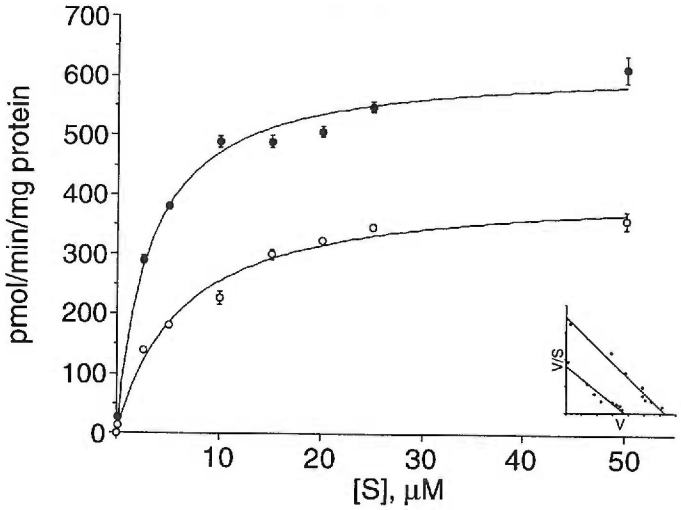
NET


700
600
500
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300
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100
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pmol/min/mg protein
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50
[S], µM
V/S
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Table 1. **Comparison of Transport Kinetics**

		-PMA	+PMA	% Change
DAT	K_T*	7.9 ± 0.8	8.5 ± 1.3	+ 7.1 ± 0.9
	V_{max}**	1012 ± 63	449 ± 31	- 55.6 ± 3.6
GAT3	K_T	5.6 ± 1.3	6.1 ± 2.1	+ 8.2 ± 1.9
	V_{max}	913 ± 34	480 ± 20	- 47.4 ± 1.5
NET	K_T	3.2 ± 0.4	3.8 ± 0.9	+ 15.8 ± 1.2
	V_{max}	578 ± 17	352 ± 18	- 39.1 ± 1.3

* μM
** pmol/min/mg protein

has been shown to underlie the PKC-mediated downregulation of the SERT (138). However, this has not been formally demonstrated for other members of the Na^+/Cl^- transporter family. Therefore, cell surface biotinylation was used to assess the amount of carrier protein found at the surface of MDCK cells expressing the transporters following incubation with PMA. MDCK-DAT, MDCK-GAT3, and MDCK-NET cells were treated with 100 nM PMA, or vehicle alone for 1 h. Additional cells expressing each transporter were exposed to PMA for 1 h, washed extensively, and then incubated without PMA for another 3 h prior to cell surface biotinylation. Surface proteins were labeled with a membrane impermeant biotin derivative and recovered with Neutravidin-agarose resin. Biotinylated proteins were separated by SDS-PAGE, and visualized with transporter specific antisera.

Incubation with PMA significantly reduced the amount of transporter protein available for biotin labeling at the cell surface (Fig. 5). The intensity of the bands in PMA treated lanes was the same immediately after the 1 h PMA treatment as after the 3 h washout of PMA, suggesting that within the time frame of this experiment, the transporter protein does not return to the cell surface. Only biotinylated transporter proteins were recovered with the Neutravidin resin, as evidenced by lack of immunoreactivity in cells that were not biotin labeled.

The presence of unlabeled transporters was also observed in the unbiotinylated fraction of all samples following Neutravidin recovery. The unlabeled transporter protein consisted almost exclusively of more rapidly migrating partially glycosylated species (data not shown), suggesting that only mature proteins that had reached the cell surface are represented in the biotinylated fraction. The non-specific bands recognized by the α-DAT antisera also appear to be biotinylated (refer to Fig. 1). The upper band seen with the α-GAT3 antisera is believed to be the result of dimer formation occurring during processing of the protein for SDS-PAGE. Note that there are equivalent amounts

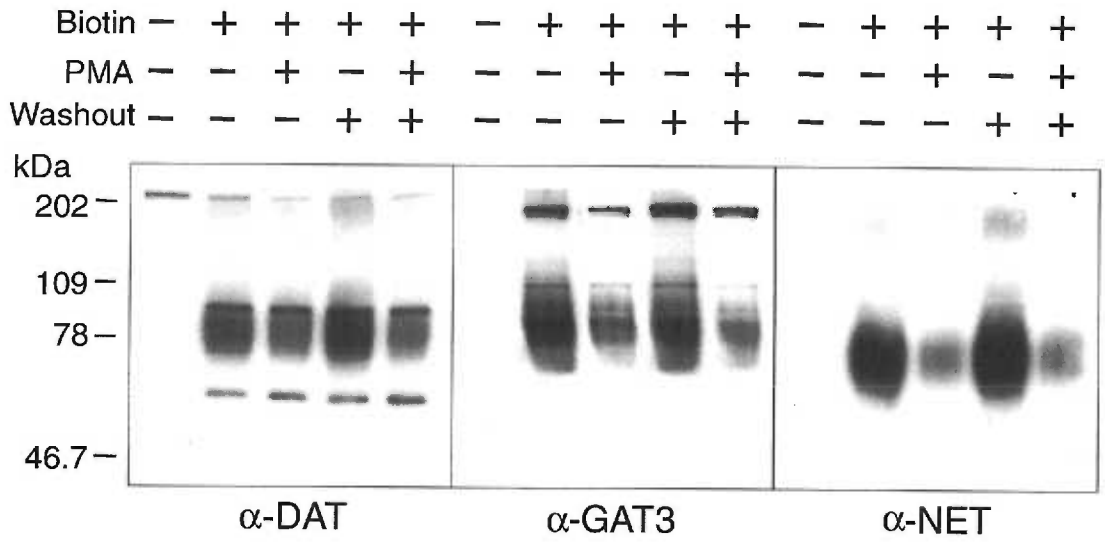

Figure 5. **Surface expression of DAT, GAT3, and NET are reduced following incubation with PMA.** MDCK-DAT, MDCK-GAT3, and MDCK-NET cells were treated with 100 nM PMA or DMSO vehicle for one h prior to cell surface biotinylation. Following PMA treatment, additional cells were washed extensively, and incubated at 37° C for an additional 3 h (washout) before biotinylation. PMA-treated or control cells were incubated in either the presence or absence of NHS-SS-Biotin at 4°C. Biotinylated membrane proteins were isolated with NeutrAvidin beads, separated by SDS-PAGE, and transferred to membrane. The blots were probed with α-DAT or α-GAT3 antisera, or with affinity purified α-NET antibody. Representative blots from two separate experiments are shown. Prestained molecular weight standards were run in parallel.

of the upper band in comparison to the lower (monomer) band in all lanes.

Taken together, these data indicate that there is a loss of mature transporter protein at the cell surface following incubation with PMA, and that the transporters do not return to the cell surface when PMA is withdrawn.

Neurotransmitter Transporters are Internalized in Response to PMA - To further examine alterations in the cellular distribution of the transporters, indirect immunofluorescence was used to determine the localization of the neurotransmitter transporter proteins after treatment with PMA. MDCK-DAT, MDCK-GAT3, and MDCK-NET cells were plated on at low density on coverslips and maintained under normal growth conditions for 2-3 days prior to processing for immunofluorescence. The cells were exposed to 100 nM PMA for 1 h, and the transporter proteins were visualized using transporter specific antisera.

The subcellular localization of the transporters after treatment with phorbol ester was dramatically different than that seen in control or untreated cells (Fig. 6A). The immunofluorescent signal was concentrated in brightly stained vesicles within the cytoplasm, instead of being primarily localized to the plasma membrane as in control cells. A small amount of perinuclear fluorescence was also observed in control cells, suggesting the presence of immature protein transiting through the secretory pathway. Background staining of the nucleus was present in cells stained with α-GAT3 antisera, and was also seen in MDCK-CMV5 cells probed with the same antisera (data not shown).

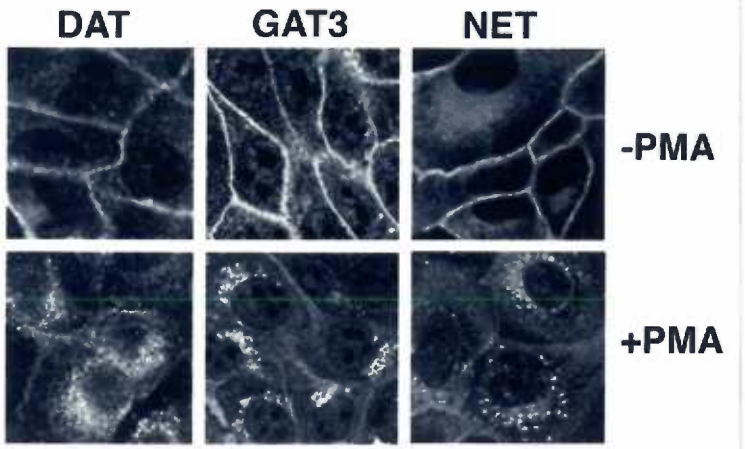

Figure 6. **DAT, GAT3, and NET are internalized in response to PMA.** *A*, MDCK-DAT, MDCK-GAT3, and MDCK-NET cells were treated with DMSO vehicle or 100 nM PMA for 1 h prior to processing for immunofluorescence. *B*, MDCK-NET cells were treated for 1 h with DMSO vehicle, 10 μM forskolin, 100 nM 4-α-phorbol, 100 nM staurosporine, or 100 nM PMA, or pretreated for 15 min with 100 nM staurosporine prior to the addition of PMA. The cells were fixed, permeabilized, and incubated with α-DAT, α-GAT3, or α-NET antisera, followed by incubation with fluorescein-conjugated secondary antibodies. Labeled cells were imaged with a BioRad MRC1000 krypton/argon scanning confocal microscope. Optical sections were taken at approximately the midway point through the cells.

DISCUSSION

PKC-mediated inhibition of uptake activity has been observed for the Na^+/Cl^--dependent family of neurotransmitter transporters in both native preparations (6,25,38,54,85,111,114,123,151,171-173,180) and a number of heterologous expression systems (39,70,81,123,186,189). The MDCK cell line was chosen as a model system for examining the regulation of the Na^+/Cl^- transporters in this study because they have proven useful for investigating the properties of proteins normally expressed in the central nervous system. The correlation between the localization of polarized proteins in neurons and MDCK cells suggests that at least some of the cellular proteins involved in protein trafficking are shared between these cell types. Furthermore, the MDCK cell line was chosen as a model system because two members of the Na^+/Cl^- transporter family, the betaine-GABA transporter (BGT-1) (184), and the taurine transporter (TAUT) (176) are endogenously expressed. The native expression of members of this gene family in MDCK cells suggests that this cell type might express accessory proteins that are involved in the regulation of transporter activity, although the identity of such proteins remains unknown.

When exogenously expressed in MDCK cells, the DAT, GAT3 and NET are all downregulated in response to the activation of PKC (Fig. 2). Interestingly, the activity of both the endogenously expressed BGT-1 and TAUT are inhibited by PMA in a similar manner. The V_{max} of the BGT-1 and TAUT are decreased by 43.7% and 56% respectively following incubation with PMA (97±8.2 vs. 54±6.0 for BGT-1 and 311±30 vs. 137±16 for TAUT), while the K_T remains unchanged (112±6.0 vs. 115±18 for BGT-1 and 11±3 vs. 12±4 for TAUT) (data not shown). Thus, PMA inhibits the activity of all of Na^+/Cl^- transporters examined when expressed in MDCK cells. Several lines of evidence suggest that PMA-mediated inhibition is a common property of the transporters

rather than being attributable to MDCK cell expression. First, the Na^+/Cl^- transporters are universally inhibited in response to PMA, regardless of the cell type in which they are expressed (25,38,54,61,70,81,96,138,151,153,172,173,186,189). The only exception is GAT1, which when expressed in *Xenopus* oocytes, has been reported to be upregulated in response to PMA (39). However, in a conflicting report GAT1 activity was inhibited by PKC activation in oocytes (123). The apparent discrepancy between these two studies may lie in the method by which PMA was applied. GAT1 activity was decreased when PMA was bath applied, and increased when PMA was injected directly into the cell. While the differences in these findings has not been fully explained, introducing PMA directly into the cytoplasm may activate a different subset of PKC isoforms than external application. Internalization of the transporters after PKC stimulation does not appear to be the result of a generalized increase in endocytosis because localization of endogenously expressed e-cadherin was not altered in MDCK cells following incubation with PMA (data not shown).

The decrease in transport activity observed following activation of PKC (Fig. 2) could result from direct effects on the transporters, or from indirect actions of the kinase, for example on proteins that are involved in maintaining the electrochemical driving force of sodium ions. Although the activity of the Na^+/K^+-ATPase is inhibited in response to phorbol esters (20), perturbation of the electrochemical gradient of Na^+ does not appear to be responsible for the decrease in transporter activity. The accumulation of [^{3}H]alanine, through a separate Na^+-dependent mechanism, is unaffected by the addition of PMA in MDCK cells expressing the Na^+/Cl^- transporters (data not shown).

The inhibition of the Na^+/Cl^- transporters by PMA can be directly attributed to the activation of PKC. The inactive α isomer of PMA neither stimulates PKC activity, nor effects a change in transporter activity (Fig. 2). Inhibition of the carriers by PMA is both time- and concentration-dependent (Fig. 3), and can be effectively blocked by the PKC

inhibitor staurosporine (Fig. 2). Although the primary sequences of the Na^+/Cl^- transporters contain multiple consensus sites for phosphorylation by PKC, mutation of these sites in the GAT1 and glycine transporters did not reduce PMA-mediated modulation (39,152). Therefore, we cannot rule out the possibility that the phosphorylation events which lead to transporter downregulation are due to the actions of an unknown kinase, which is itself activated through phosphorylation by PKC.

Perhaps the most well known example of regulated trafficking of an active transport system is the translocation of the facilitated glucose transporter GLUT4 in response to insulin. Incubation of adipocytes expressing GLUT4 with insulin results in the rapid redistribution of the transporter from subcellular compartments to the plasma membrane (41,169). The insulin-induced translocation of GLUT4 is fully reversible, and when insulin stimulation is terminated GLUT4 returns to an intracellular compartment (41). The Na^+/Cl^- transporter GAT1, which can be recruited to the cell surface in response to PMA, has also been shown to return to an intracellular localization when PKC activity is inhibited (39). In contrast, an earlier report suggested that internalization of the DAT was irreversible (189). Our observation that the internalized DAT, GAT3, and NET do not return to the cell surface when exposure to PMA was discontinued (Fig. 5) confirms this finding.

Following endocytosis, membrane proteins are sorted into two distinct cellular pathways. From the endosome, glycoproteins can be delivered to the recycling pathway for return to the cell surface, or to lysosomal compartments for degradation (for review see 113). The fate of Na^+/Cl^- transporters would largely be determined by the cellular pathway into which they are directed following internalization. Under normal conditions, the transferrin receptor is constantly recycled between the cell surface and subcellular compartments. Activation of PKC increases the rate of endocytosis of the transferrin receptor, resulting in a decrease in the number of the receptor molecules at the cell surface

(157). However, recycling of the transferrin receptor to the cell surface is unaffected, and eventually the receptor molecules return to the plasma membrane. In contrast, the EGF receptor, which like the Na^+/Cl^- transporters is internalized in response to PMA, is directed to the lysosome, where it is subsequently degraded (15). Although the Na^+/Cl^- transporters do not return to the cell surface after PKC stimulation, the fate of the carriers following PMA induced internalization has yet to be determined.

PKC-mediated inhibition of the Na^+/Cl^- transporters would be expected to enhance and prolong synaptic transmission following neurotransmitter release *in vivo*. The effects of transporter downregulation on postsynaptic receptors may be similar to those associated with pharmacologic inhibition of the transporters. For example, the euphoria and hyperlocomotion associated with the cocaine-mediated inhibition of the catecholamine transporters. Although no studies have directly demonstrated regulation of transporter activity by PKC in the brain, our results suggest that such PKC-mediated effects are possible and should be the subject of future investigations.

Regulated Trafficking of the Human Dopamine Transporter: Clathrin-mediated Internalization and Lysosomal Degradation in Response to Phorbol Esters

Gwynn M. Daniels‡¶ and Susan G. Amara‡§¶

From the ‡Department of Cell and Developmental Biology, §Howard Hughes Medical Institute, and the ¶Vollum Institute, Oregon Health Science University, Portland, Oregon 97201.

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SUMMARY

The dopamine transporter plays an essential role in the modulation of dopaminergic neurotransmission by mediating the reuptake of dopamine into presynaptic neurons. In cells expressing the dopamine transporter, activation of protein kinase C by phorbol esters results in a significant reduction in dopamine uptake. This phorbol ester-mediated inhibition of dopamine transport is associated with a decrease in V_{max}, although the apparent affinity of the transporter for dopamine remains unchanged. Using a green fluorescent protein-tagged dopamine transporter stably expressed in MDCK cells, we show in live cells that the decrease in transporter activity is caused by the rapid internalization of carriers from the plasma membrane. This redistribution of the transporter is specific to phorbol ester activation, and is unaffected by the presence of either substrates or inhibitors of the carrier. Upon addition of phorbol esters, transporters at the cell surface are rapidly endocytosed through a clathrin-mediated and dynamin-dependent mechanism into early endosomes, where they colocalize with transferrin. The internalized carrier is targeted to the endosomal/lysosomal pathway, and is completely degraded within 2 h of protein kinase C activation. Phorbol ester-mediated alterations in the trafficking of the dopamine transporter may serve as a mechanism for controlling extracellular dopamine levels in the central nervous system.

INTRODUCTION

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 psychostimulant drugs, which block the reuptake of released dopamine, resulting in an increase in synaptic dopamine levels (reviewed in 5). Functional loss of dopamine transport, either through pharmacological inhibition (149) or genetic knockout (51), 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 (51,79,160). 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 (38,70,81,180,186,189). 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 (70,180). 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 non-transported inhibitors of the DAT (81,186,189). 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 (189). Activation of PKC has also been suggested to result in changes in the subcellular localization of hDAT (137). However, high intracellular levels of DAT associated with transient overexpression systems made it difficult to evaluate the difference in transporter distribution by indirect immunofluorescence. While 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 . In order 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.

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 (PMSF) was obtained from Gibco/BRL. Forskolin, 3-isobutyl-1-methylxanthine (IBMX), PMA, 4-α-phorbol, staurosporine, and bafilomycin A1 were purchased from Calbiochem. Anti-actin antibody was from Boehringer Mannheim, and lactacystin was obtained from Corixa Corp.

Plasmid construction, development of stable lines, and cell culture - The hDAT cDNA was inserted between the *Kpn 1* and *Xba 1* sites of pEGFP-C1 (Clontech), creating the plasmid pEGFP-hDAT, which expresses a protein with EGFP directly fused to the amino terminus of the hDAT.

MDCK cells (ATCC) were grown to approximately 80% confluence in 35mm tissue culture dishes, and exposed to a solution containing 1.5 μg plasmid DNA (pEGFP-hDAT or pEGFP-C1) and 40 μg LIPOFECTAMINE™ reagent (Gibco/BRL) in serum-free DMEM for 5 h, after which the DNA/cationic lipid solution was replaced by DMEM supplemented with 10% FCS (growth medium) containing 10 U/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 (Gibco/BRL). 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 (133).

Briefly, uptake was initiated by the addition of 100 nM [^{3}H]-dopamine (New England Nuclear), with or without unlabeled dopamine or inhibitor, and was allowed to continue for 10 min at room temperature. Background uptake 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.

Cell surface biotinylation and Western blotting - Cell surface biotinylation was performed as described (see appendix). MDCK-GFP:DAT cells were grown to near confluence in 6-well plates, and incubated with biotinylation buffer (150 mM NaCl, 2 mM $CaCl_2$, 10 mM triethanolamine, pH 7.5) containing 2 mg/ml NHS-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 10 min at room temperature in 2X SDS sample buffer followed by a 30 min incubation at 37°C.

For immunoblotting, MDCK-GFP:DAT cells were treated as indicated, and extracted in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5, 1% Triton X-100) with or without protease inhibitors (2 mM PMSF and 2 μg/ml each aprotinin, antipain, chymostatin, leupeptin, and pepstatin A). Conditions for *in vitro* endoglycosidase treatment were as described (119). Cell extracts were diluted in 2X SDS sample buffer and incubated as above.

Proteins were separated by SDS-PAGE, transferred to membranes, and Western blots were performed as described (119). Blots were probed with polyclonal anti-GFP antisera (Clontech) diluted 1:5000.

Fluorescence microscopy - Cells grown to confluence on glass coverslips were treated as indicated, rinsed with PBS, and fixed in freshly prepared 4% paraformaldehyde in PBS for 20 min at room temperature. Following fixation, the cells were washed in

PBS and the coverslips were mounted on glass slides with ProLong (Molecular Probes) antifade reagent.

For indirect immunofluorescence, paraformaldehyde fixed cells were washed in PBS, and then blocked and permeabilized in 1% BSA, 2% normal horse serum (blocking buffer) containing 0.5% Triton X-100 (for DECMA-1 antibody) or 0.075% saponin (for AC17 antibody) for 20 min at room temperature. The fixed and permeabilized cells were washed, and incubated for 1-3 h at room temperature with the primary antibody diluted in blocking buffer. After incubation with primary antibody, the cells were washed with PBS and incubated for 1 h in blocking buffer containing secondary antibody conjugated to lissamine rhodamine or rhodamine red-X (Jackson). Following incubation with secondary antibody, the cells were washed extensively in PBS, and mounted on glass slides as described above.

Vaccinia virus infection and transferrin uptake- Cells grown to confluence on glass coverslips were infected with wild-type or recombinant vaccinia virus. The cells were infected at a multiplicity of infection (M.O.I.) of 5 for 30 min at room temperature in PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$. The inoculum was removed, and incubation was continued in normal growth medium.

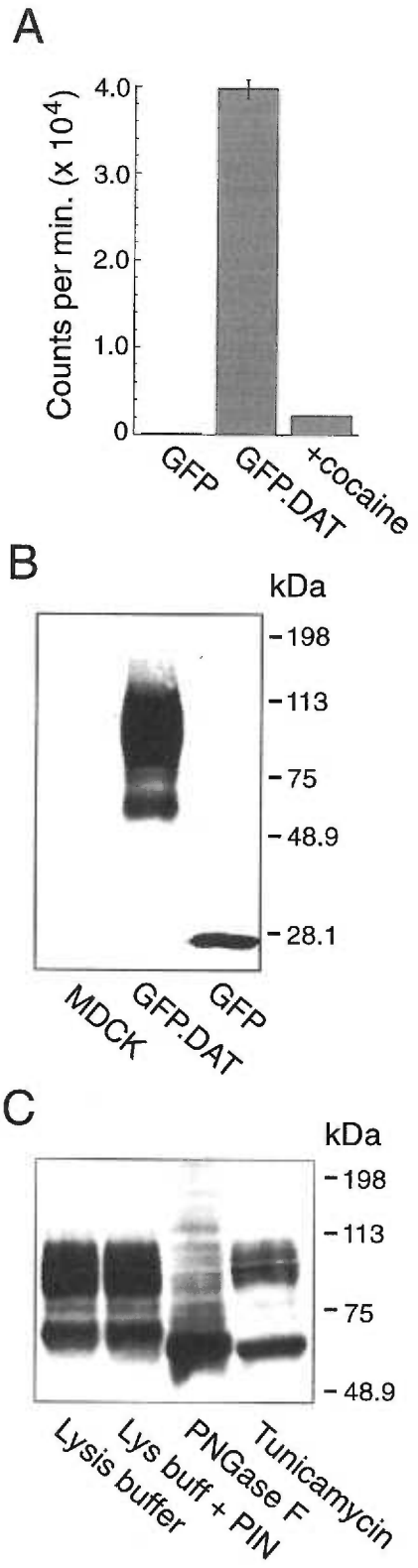
Canine apo-transferrin (Sigma) was loaded with iron as described (131), and labeled with ALEXA 468 (Molecular Probes) according to the manufacturer's recommendations. Cells were incubated with 112.5 ng/ml labeled transferrin for the times indicated.

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 inhibited 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 kD (Fig. 1B) only in the GFP-DAT expressing cells. This apparent molecular weight is approximately 27 kD greater than that reported for wild-type hDAT (181), consistent with the presence of the GFP tag. Correspondingly, cells expressing only GFP exhibited a single immunoreactive species of approximately 27 kD. A second band, centered at approximately 66 kD, was observed in GFP:DAT expressing cells, but not in cells expressing GFP alone. To determine the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Counts per min. (x 10^4)
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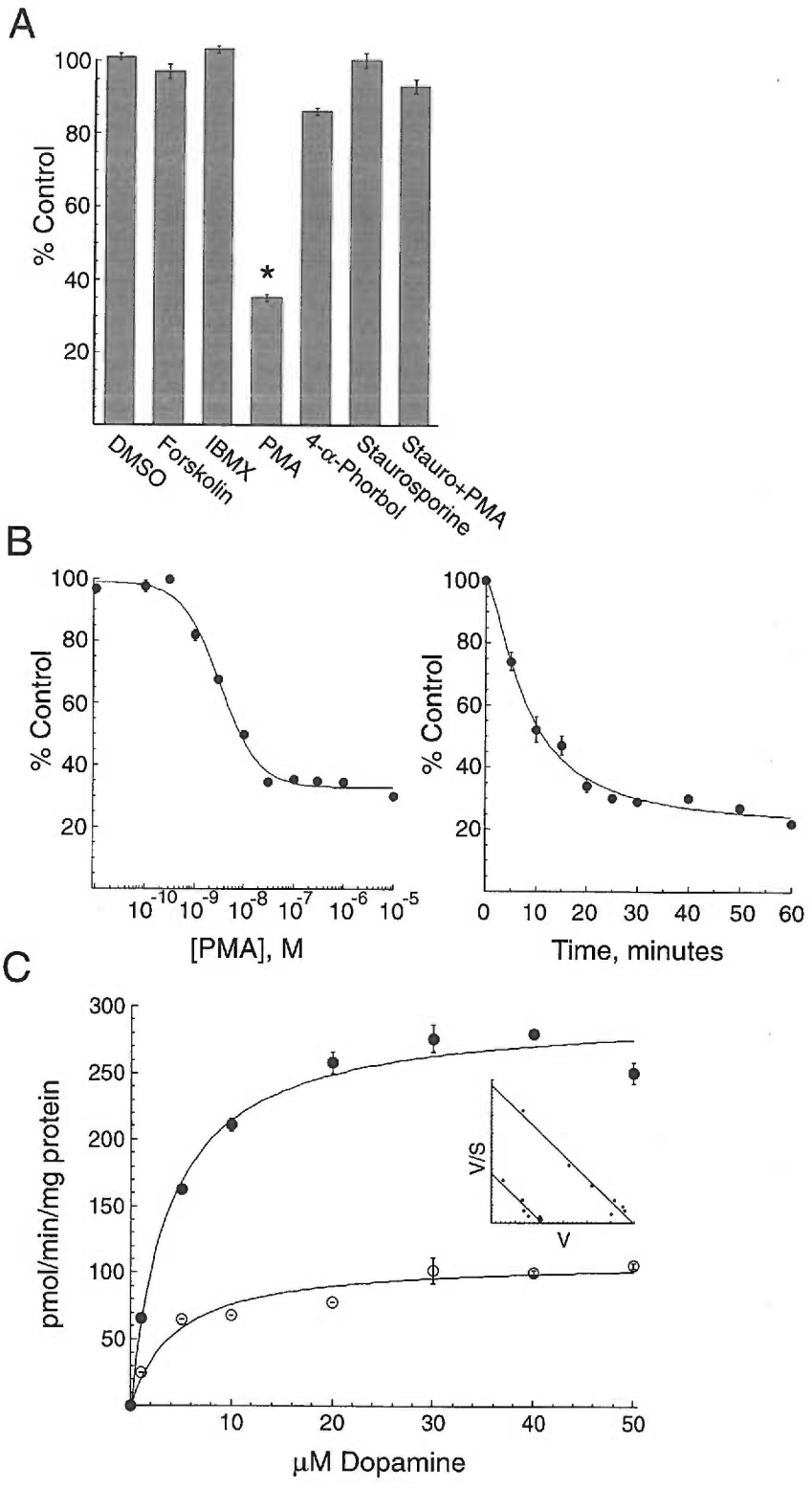
3.0
2.0
1.0
0
GFP
GFP.DAT
+cocaine
B
kDa
198
113
75
48.9
28.1
MDCK
GFP.DAT
GFP
C
kDa
198
113
75
48.9
Lysis buffer
Lys buff + PIN
PNGase F
Tunicamycin

Fig. 1. **Characterization of MDCK cells stably expressing GFP:DAT.** *A*, Uptake of radiolabeled dopamine in MDCK-GFP (GFP) and MDCK-GFP:DAT (GFP:DAT) cells expressed as cpm per well ± s.e.m. Cells were incubated with [^{3}H]-dopamine in the presence or absence of cocaine. *B*, Western blots of cell extracts from wild-type MDCK, MDCK-GFP, or MDCK-GFP:DAT cells. Total protein (25 μg) from cell lysates was probed with antisera to GFP. *C*, Deglycosylation of GFP:DAT. MDCK-GFP:DAT cells were incubated in the absence (lanes 1-3) or presence (lane 4) of tunicamycin and solubilized in lysis buffer alone (lanes 1 and 4), or in lysis buffer containing protease inhibitors (PIN) (lane 2). Lysates from an equivalent number of cells were incubated with PNGase F (lane 3).

identity of this band, MDCK-GFP:DAT cells were incubated for 16 h in the presence of tunicamycin to block N-linked glycoslyation. Immunoblots of lysates from tunicamycin-treated cells showed significantly reduced levels of the 108 kD band (Fig. 1C). The lower band appeared to migrate at 58 kD, 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 (PNGase F) suggesting that it represents the unglycosylated form of the DAT. Furthermore, cells solubilized in lysis buffer containing a cocktail 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 weight 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 (38,70,81,180,186,189). 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 to 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, while 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).


A
100
80
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DMSO
Forskolin
IBMX
PMA
4-α-Phorbol
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[PMA], M
100
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0 10 20 30 40 50 60
Time, minutes
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300
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200
150
100
50
pmol/min/mg protein
V/S
V
10 20 30 40 50
μM Dopamine

Fig. 2. **PMA-mediated inhibition of uptake activity in MDCK-GFP:DAT cells.** *A*, Uptake of [^{3}H]-dopamine in MDCK-GFP:DAT cells following 20 min incubation at 37°C with vehicle alone (DMSO), 10 μM forskolin, 100 μM IBMX, 100 nM PMA, 100 nM 4-α-phorbol, 100 nM staurosporine, or 100 nM PMA after 15 min preincubation with 100 nM staurosporine. Values are the percentage of uptake activity compared to untreated cells ± s.e.m. *B*, Time- and concentration-dependence of PMA-induced inhibition of uptake activity. Accumulation of [^{3}H]-dopamine after treatment with 10 pM to 10 μM PMA for 20 min, or with 100 nM PMA for 0-60 min at 37°C. Data are expressed as a percentage of uptake activity in cells treated with vehicle alone ± s.e.m. *C*, Effect of PMA treatment on transport kinetics. MDCK-GFP:DAT cells were treated with DMSO (filled circles) or PMA (open circles) prior to uptake assay. The cells were exposed to [^{3}H]-dopamine combined with increasing concentrations of unlabeled dopamine. Uptake velocity is expressed as pmol of dopamine/min/mg of total protein ± s.e.m. Eadie Hofstee transformation of the same data are shown (inset). Values for V_{max} and K_T were calculated by nonlinear regression analysis of the untransformed data. All data shown are representative results of at least 3 separate experiments performed in triplicate. * p <.005 by Student's paired t test.

Dopamine uptake was also examined after incubation with PMA at concentrations ranging from 10 pM to 10 μM. Half maximal inhibition (IC_{50}) 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 $T_{1/2}$ 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 (V_{max} = 110 ± 6.5 versus 296 ± 10 pmol/min/mg of protein). However, the apparent affinity of the transporter for dopamine remained unchanged, with a K_T 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 (K_T = 3.9 ± 0.9 μM) stably expressed in MDCK cells (data not shown). Affinity of the transporter for substrate was also comparable to that determined for the wild-type hDAT in other model systems (186,189). 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 (137,186,189).

Subcellular distribution of GFP:DAT. A decrease in V_{max} , 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_{max}, 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 MDCK.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, while staurosporine alone had no effect on transporter localization. These observations are consistent with results seen for uptake activity under the same conditions (Fig. 2A).

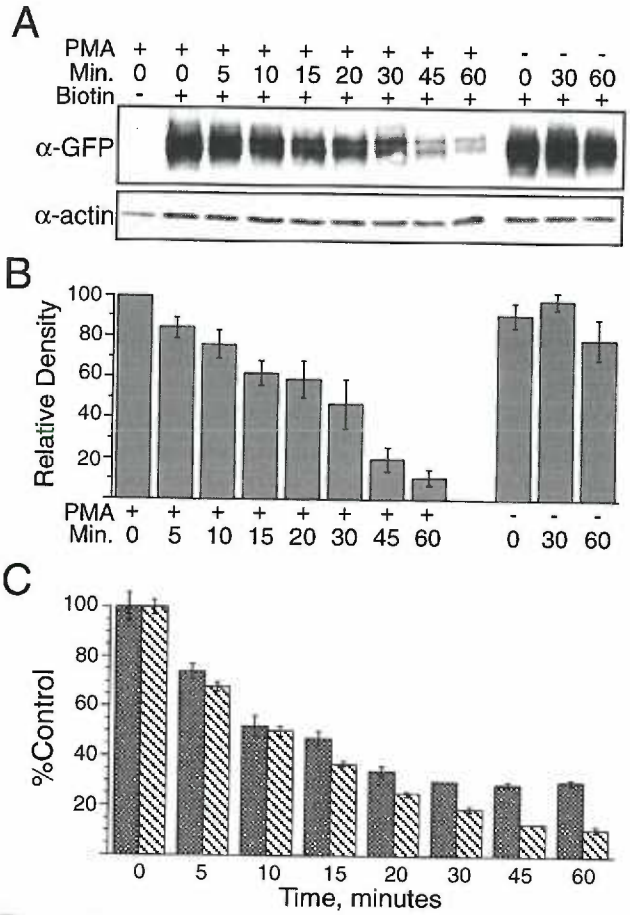
In a recent report, the γ-aminobutryic acid (GABA) transporter GAT1, was shown to undergo changes in cellular distribution after incubation with GABA or with SKF89976A, a specific inhibitor of GAT1 (19). To determine whether the presence of either substrate or inhibitor had an effect on GFP:DAT localization, MDCK-GFP:DAT cells were stimulated with PMA following 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 (98), incubation of MDCK-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 MDCK-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 kD 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 one h (Fig. 4B). However, no significant change was seen in cells treated with vehicle alone. Because previous results suggested that no more than 70% of uptake activity was


A
PMA + + + + + + + + + - - -
Min. 0 0 5 10 15 20 30 45 60 0 30 60
Biotin - + + + + + + + + + + +
α-GFP
α-actin
B
Relative Density
100
80
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40
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PMA + + + + + + + + - - -
Min. 0 5 10 15 20 30 45 60 0 30 60
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100
80
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0 5 10 15 20 30 45 60
Time, minutes
D
Control
+PMA
+CHX +PMA
0'
30'
60'

Fig. 4. **Surface expression of GFP:DAT is reduced in a time-dependent manner after PMA treatment.** *A*, MDCK-GFP:DAT cells were treated with cycloheximide for 4h and then incubated with PMA or vehicle alone for 0-60 min prior to cell surface biotinylation. Shown is a representative blot from one of three separate experiments. The blot was stripped and reprobed with anti-actin antibody to confirm equal loading in each lane. *B*, Densitometric analysis of cell surface biotinylation following PMA treatment. Equal areas in each lane from 3 separate blots were evaluated for pixel density using NIH Image. All values are normalized to actin control, and are expressed as a percentage of the 0 timepoint ± s.e.m. *C*, Comparison of uptake activity with and without cycloheximide pretreatment. MDCK-GFP:DAT cells were incubated in the presence of cycloheximide (hatched bars) or vehicle control (stippled bars) followed by treatment with PMA for 0-60 min. Values are the percentage of uptake compared to DMSO treated controls ± s.e.m. Shown are representative results of 3 separate experiments performed in triplicate. *D*, Live cell images of MDCK-GFP:DAT cells treated with PMA or vehicle control in the presence or absence of cycloheximide. MDCK-GFP:DAT cells were incubated in the presence of cycloheximide or vehicle alone, transferred to a chamber apparatus (Medical Systems Corp., Greenvale, NY), and maintained at 33°C in growth medium buffered with 25 mM HEPES. Confocal images were collected over a period of 60 min following treatment with PMA or vehicle control.

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, while 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. MDCK-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 the cell surface (Fig. 4D), consistent with what was observed in fixed control cells (refer to Fig. 3). A gradual increase in punctate intracellular fluorescence concurrent with a decrease in the fluorescent intensity at the plasma membrane was observed over a 30 min exposure to PMA, after which the distribution of the transporter remained unchanged for the duration of the experiment. In contrast, GFP:DAT remained localized to the plasma membrane of cells that were exposed to vehicle alone. Additional MDCK-GFP:DAT cells were preincubated 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 endocytotic event occurs. The GTPase dynamin associates with CCPs, and plays an essential role in the budding of endocytic vesicles (66,161). We utilized a dominant negative mutant of dynamin 1 to effectively block clathrin-mediated endocytosis. MDCK-GFP:DAT cells were infected with recombinant vaccinia viruses expressing either wild-type dynamin 1 or K44E, a dominant negative mutant of dynamin 1 (65). 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 dynamin exhibit no accumulation of transferrin, as expected based on the results of previous studies (65,112,179). 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.

Double-labeling of GFP:DAT and markers of intracellular compartments 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

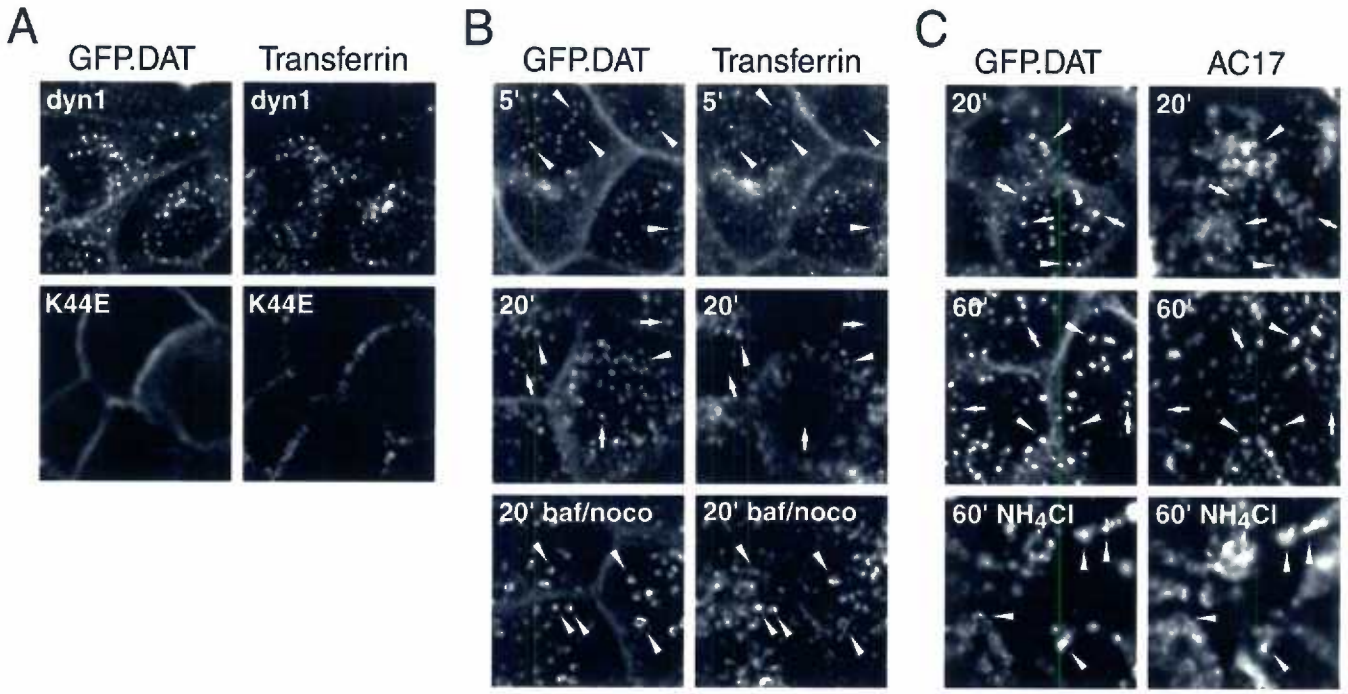

A
GFP.DAT
Transferrin
dyn1
dyn1
K44E
K44E
B
GFP.DAT
Transferrin
5'
5'
20'
20'
20' baf/noco
20' baf/noco
C
GFP.DAT
AC17
20'
20'
60'
60'
60' NH4Cl
60' NH4Cl

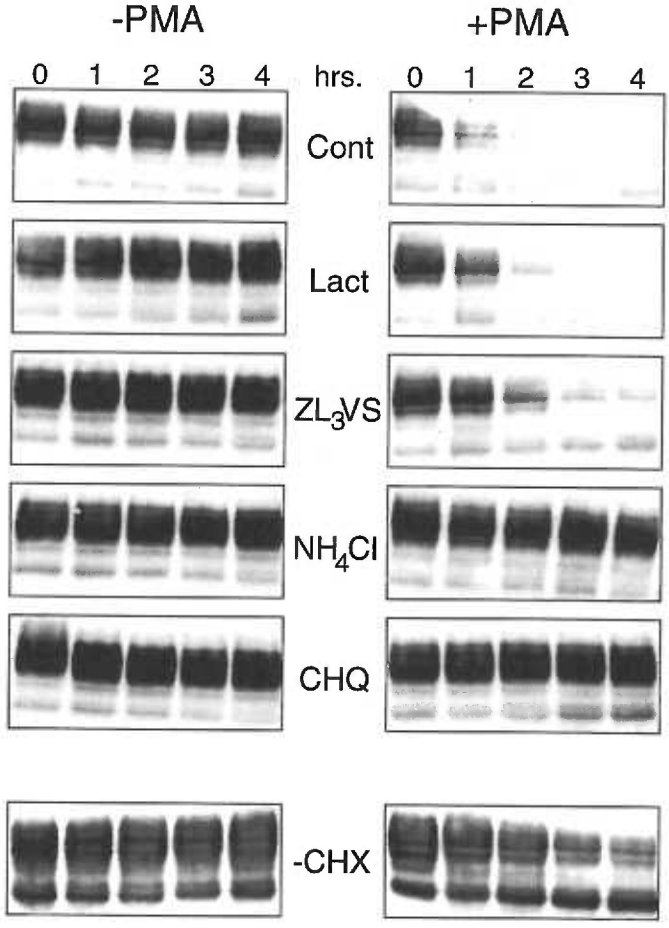
Fig. 5. **Endocytosis of GFP:DAT in response to PMA.** *A*, MDCK-GFP:DAT cells were infected with recombinant vaccinia viruses expressing wild-type dynamin 1 or the dominant negative mutant K44E. 8 h post-infection, the cells were incubated in the presence of PMA and Alexa 568-labeled canine transferrin. *B*, Colocalization of GFP:DAT with early endosomes. MDCK-GFP:DAT cells were preincubated with labeled transferrin at 4°C and then incubated with PMA for 5 min at 37°C (upper panels), or incubated for 20 min at 37°C with PMA in the presence of transferrin (center panels), or with PMA and transferrin after preincubation with nocodazole and bafilomycin A1 (lower panels). *C*, Colocalization of GFP:DAT with lysosomes. MDCK-GFP:DAT cells were treated for 20 min with PMA (upper panels) or for 60 min with PMA in the absence (center panels) or presence (lower panels) of 50 mM NH_4Cl. Lysosomes were labeled with monoclonal antibody AC17. Arrows indicate areas of divergent fluorescence, and arrowheads indicate areas of overlapping fluorescence.

transferrin (Fig. 5B). However, if the cells were preincubated with bafilomycin A1 and nocodazole, conditions which have been shown to block maturation of early endosomes (42), 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 timepoint following application of PMA. In order to get 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 (117). After 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 (23). Therefore, lysosomal acidification and the activity of lysosomal proteases were blocked by incubating the cells with NH_4Cl 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 Following 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, TCA precipitation of radiolabeled proteins showed that greater than 97% of protein synthesis was inhibited, and protein synthesis did not recover during the timecourse 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 timepoints following removal of PMA from the culture medium. One h after PMA treatment, there is a distinct reduction in GFP:DAT protein as compared to control cells exposed to DMSO vehicle alone, or to the starting time point (immediately following the 20 min PMA incubation) (Fig. 6). After 2 h, GFP:DAT protein is barely visible in PMA-treated cells, and by 3 h it is completely absent. GFP:DAT levels remain unchanged, however, in cells incubated with the lysosomotropic amines NH_4Cl or chloroquine, either with or without PMA stimulation. In contrast, lactacystin or the vinyl sulfone ZL_3VS, specific inhibitors of proteasomal degradation, failed to block PMA-mediated degradation of GFP:DAT. When protein synthesis is not inhibited by cycloheximide, GFP:DAT levels are significantly reduced after incubation with PMA, but the protein is 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 is 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.


-PMA
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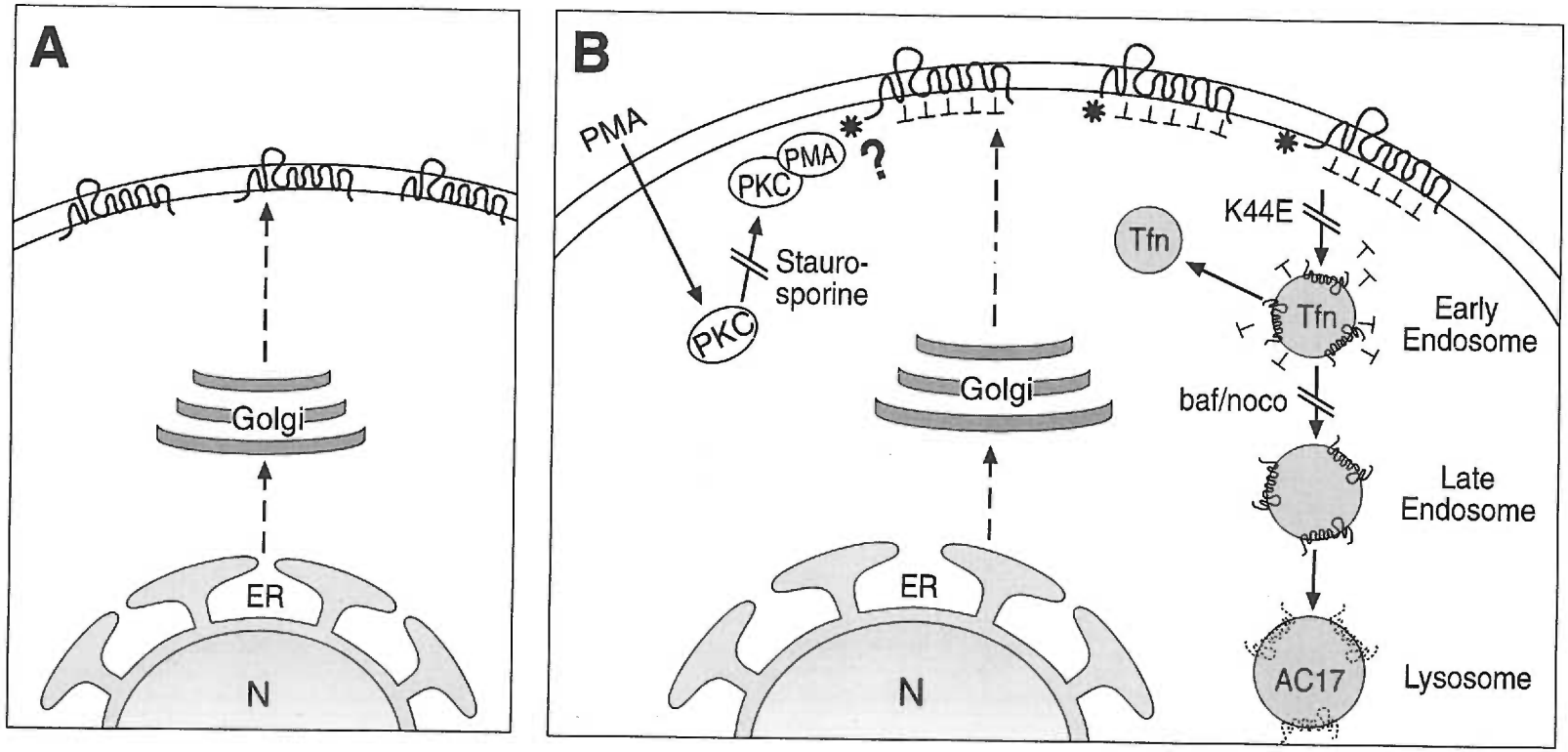
0 1 2 3 4
hrs.
0 1 2 3 4
Cont
Lact
ZL3VS
NH4Cl
CHQ
-CHX

Fig. 6. **Lysosomal degradation of GFP-DAT.** MDCK-GFP:DAT cells were preincubated for 3 h with or without cycloheximide (CHX), and for 1 h with either 25 μM lactacystin (Lact), 35 μM ZL_3VS, 50 mM NH_4Cl, or 100 μM chloroquine (CHQ), in the continued presence of cycloheximide. The cells were then incubated with 100 nM PMA or vehicle control, washed, and incubation was continued in the presence of the protease inhibitors and cycloheximide.

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 (9,25,39,81,114). A number of studies have implicated internalization of the transporters as a possible means of modulating transporter activity (9,137,138,186,189). A recurrent observation has been that PKC activation has little impact on substrate affinity, but significantly decreases V_{max}, consistent with either a decrease in the catalytic rate of the carrier, or a reduction in the number of transporter molecules at the cell surface. 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 (9,138). Recent reports have suggested that the dopamine and norepinephrine transporters are redistributed after PKC activation when examined by indirect immunofluorescence (9,137). However, the results of these studies were obscured by high intracellular levels of transporter, making interpretation of the data difficult. Thus, the cellular pathways involved in the PMA-mediated translocation of the DAT, and the fate of the internalized protein have not been determined.

We provide 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. Based on the results presented, we have developed a model for PMA-mediated regulation of the DAT (represented in Fig. 7). Under control conditions (Fig. 7A), the majority of DAT protein is found at the cell surface. Only the


A
Golgi
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sporine
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Golgi
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N
K44E
Tfn
Tfn
Early
Endosome
baf/noco
Late
Endosome
AC17
Lysosome

Fig. 7. **A model of PMA-mediated regulation of the hDAT.** *A*, Trafficking of the hDAT under control conditions. *B*, Trafficking of the hDAT after PKC activation. The PMA-mediated activation of PKC causes a staurosporine-sensitive phosphorylation event leading to the redistribution of the carrier. The hDAT undergoes an increase in dynamin-dependent endocytosis through CCPs. The transporter is rapidly internalized into early endosomes, where it colocalizes with transferrin (Tfn). Late endosomes containing the GFP:DAT segregate from vesicles containing Tfn, and enter the lysosomal pathway. Targeting of the hDAT to AC17 labeled lysosomal compartments results in complete degradation of the transporter. Nucleus (N), endoplasmic reticulum (ER), PO^4 (*),clathrin (Z).

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).

Upon activation of PKC by PMA (Fig. 7B), PKC is translocated from the cytosol to the plasma membrane (83). Once localized to the plasma membrane, a PKC-mediated phosphorylation event leads to the rapid internalization of the transporter. Whether internalization is the result of the direct phosphorylation of the hDAT remains unclear (indicated by the question mark in Fig. 7B). 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 PMA stimulation (70,180,182). 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 PMA-mediated inhibition of transporter activity (39,152). 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 DAT requires the phosphorylation of a protein that is associated with DAT at the cell surface. However, the possibility remains that translocation is the result of direct phosphorylation of 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 following 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. Expression of this dynamin mutant has been shown to inhibit transferrin uptake primarily from the basolateral surface of polarized MDCK cells (3). However, we observed a complete loss of transferrin accumulation in MDCK-GFP:DAT cells. This may be because MDCK-GFP:DAT cells were not grown under conditions which allow for complete polarization, or because infection by vaccinia virus disrupts tight junctions to a greater degree than infection by the adenovirus vectors utilized in the previous study.

Dynamin has also been suggested to play a role in the budding of caveolae (63,121). 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 (165). 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 (155) (data not shown). In contrast, internalization of DAT was significantly, though not completely, abolished by either chlorpromazine or monodansylcadaverine, potent inhibitors of clathrin-mediated endocytosis (data not shown). These drugs were also unable to completely block transferrin uptake in the presence of PMA, perhaps due to the competing increase in transferrin receptor endocytosis that has been observed upon stimulation of PKC (82).

When first internalized, the DAT is found in early endosomes, where it colocalizes with transferrin (Fig. 5B). Over time, internalized DAT becomes segregated from transferrin, which is recycled back to the cell surface. However, when maturation of early endosomes is blocked by incubating the cells with bafilomycin A1 and nocodazole, colocalization of DAT with transferrin persists. The divergence of vesicles containing the DAT from those containing transferrin suggests that rather than recycling back to the cell surface, the internalized DAT is targeted to the lysosomal pathway. This was confirmed by the observation that at later timepoints GFP:DAT colocalizes with the resident lysosomal protein recognized by the monoclonal antibody AC17 (Fig. 5C). This colocalization was enhanced when lysosomal proteases were inhibited by the presence of NH_4Cl or chloroquine.

After internalized DAT enters the endosomal/lysosomal pathway it is targeted for degradation. When the synthesis of new protein is inhibited by cycloheximide, the level of total GFP:DAT protein decreases rapidly after exposure to PMA, and is completely absent from the cells within 2 h of translocation (Fig. 6). Consistent with the increase in lysosomal localization of the DAT seen when the cells were incubated with lysosomotropic amines, degradation of the DAT protein was completely blocked by NH_4Cl or chloroquine.

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 (137), 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 reflected 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. Recent studies indicated that both the norepinephrine transporter and GAT1 activities are down-regulated by stimulation of the muscarinic acetylcholine receptor (9,13). Although MDCK cells express muscarinic receptors (109), incubation of MDCK-GFP:DAT cells with excess carbachol or muscarine failed to produce any change in DAT activity or localization (data not shown).

PMA is known to modulate the surface expression of a number of biologically important proteins, including the EGF receptor (15), the Na^+/glucose cotransporter (183), chemokine receptors (4,190), and the surface glycoprotein CD4 (68). 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 (1). 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 (126,129). 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 103). The carboxyl terminal tail of the hDAT contains two tyrosine residues. One of these tyrosine residues, Y578, is positioned too close to a membrane spanning domain to serve as a signal for accumulation in coated pits (36). While the importance of the second tyrosine (Y593) 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 (103). Although the carboxyl tail of the hDAT does not contain an obvious dileucine motif, there is a dileucine sequence (L440, L441) 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 following 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 HIV induces the internalization and degradation of CD4 (60). This is attributed to the presence of the virally encoded protein nef (47), which associates directly with CD4 (2), and directs both the accumulation of CD4 first in CCPs (26), and then in lysosomes (130). Desensitization of the β_2-adrenergic receptor (β_2-AR) also involves internalization through its interaction with a secondary protein. In the presence of agonist, phosphorylated β_2-AR binds to the connector protein β-arrestin, which promotes the accumulation of β_2-AR in CCPs through a direct interaction with clathrin cages (56).

Redistribution of membrane proteins is proposed to play an important role in synaptic plasticity. Stimulation of long-term depression (LTD) has recently been demonstrated to induce the internalization of the AMPA-type glutamate receptor in hippocampal cultures (28). 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 following activation of PKC by phorbol esters (32,91). While the fate of the AMPA and GABAA receptors following 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.

In conclusion, this study provides evidence that in response to PKC activation mature DAT molecules found at the cell surface are rapidly internalized through clathrin- and dynamin-mediated endocytosis. Translocation of the carrier is irreversible, and is

independent of the presence of substrate or inhibitor. The internalized transporter is targeted to the lysosome where it is completely degraded.

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Discussion and Conclusions

When I first began this project, several members of the Na^+ and Cl^--dependent neurotransmitter transporter family had recently been cloned. As targets of both therapeutic drugs and drugs of abuse, the socioeconomic significance of these carriers is substantial. Therefore, much interest was generated in discovering how the Na^+/Cl^- transporters function on a molecular level. Because of the profound physiological and behavioral consequences associated with inhibition of the monoamine transporters, understanding how the activity of the transporters is regulated was of particular importance. There was some evidence at the time, from studies using synaptosomal preparations or cell lines that endogenously express the Na^+/Cl^- transporters, that transport activity was modulated by protein kinases, however the underlying mechanisms were unknown.

A wide array of cell types have been utilized as heterologous expression systems for studying the PKC-mediated regulation of the Na^+/Cl^- transporters (see table 1 of the introduction of this thesis). Although there are minor cell-type specific variations in the response of the transporters to activation of PKC, the results are in general very consistent. While any model system has potential drawbacks, the use of stable lines of MDCK cells expressing the transporters has proven useful for our examination of transporter regulation and trafficking. The use of MDCK cells expressing the GFP:DAT has been a particular advantage, because even at low expression levels, the protein is easily detected. While two other groups have demonstrated a PMA-mediated change in the subcellular distribution of the transporters by immunofluorescence, high expression levels made differences in transporter localization difficult to distinguish (9,137). The results of experiments from this study examining the localization of the transporters by

immunofluorescence also have a great deal more background fluorescence than data obtained using the GFP:DAT expressing cells. This background interference was unavoidable without the use of a GFP tag, due to the level of expression needed to detect the proteins by immunofluorescence. Therefore, more detailed studies of transporter regulation and trafficking were possible using the GFP-tagged transporter.

We find that the activity of the DAT, GAT3, and NET are inhibited by 40-60% following activation of PKC. Although the extent of inhibition varies significantly in different experimental systems, these results are well within the range of those found by other investigators. Somewhat surprisingly, the GFP:DAT was inhibited by PMA to a greater extent than the untagged DAT (66% vs. 56%). The difference in the PMA-mediated inhibition of the wild-type DAT and GFP:DAT may be due to the presence of the GFP tag. While the functional characteristics of the wild-type DAT and the GFP:DAT that were examined showed no appreciable differences, we cannot rule out the possibility that a conformational change in the transporter induced by the GFP tag alters the response to PMA. It is also possible that the difference in inhibition between the wild-type DAT and GFP:DAT represent a species-specific variation in the regulation of the DAT by PKC. The GFP tag was fused to the human DAT, while the untagged DAT used in the early experiments was the rat DAT. Although the rat and human homologs of the DAT are 92% identical, 3 of the amino acid residues in the rat homolog that are not found in the human homolog are potential sites for PKC phosphorylation. The importance of these sites to the PMA-mediated inhibition of the DAT is not known, however, mutation of the canonical PKC sites in other members of the Na^+/Cl^- transporter family does not alter PKC-mediated inhibition (39,152) There is only one other example of the same expression system being used to examine the regulation of both the human and rat homologs of the DAT. Activation of PKC inhibits both the rDAT (81) and the hDAT (137) when transiently expressed in COS cells. However, while the hDAT was

inhibited by PMA to a greater extent than the rDAT, the variability in both transfection and assay techniques makes it difficult to evaluate any species-specific differences in the regulation of the DAT.

The variation in the extent of PMA-mediated inhibition between the rDAT and the GFP:DAT may also be a function of expression level. The V_{max} of the rDAT expressing clone used in this study was 4-fold greater than the V_{max} of the GFP:DAT expressing clone, when normalized for total protein content. Therefore, the difference in the extent of inhibition between the rDAT and GFP:DAT may support our conclusion that following PMA-mediated internalization of the transporters the plasma membrane is repopulated by intracellular stores. Since GFP:DAT cells express the carrier at a lower level, less transporter protein is available to repopulate the cell surface, which may in turn reduce the amount of uptake activity observed after internalization. The effect of expression level on the extent of PMA-mediated inhibition in other systems is difficult to evaluate, because the criteria used to normalize transport assay data varies from lab to lab. The only direct comparison between expression level and PMA responsiveness is found in a report on the regulation of GAT1 (39), that also showed a decrease in the response to PMA at higher expression levels. Therefore, the level of protein expression may account for some of the variability observed in extent PKC-mediated inhibition of the Na^+/Cl^- transporters.

The translocation of substrate across the plasma membrane necessitates a change in the conformation of a transporter protein. Changes in protein conformation induced by transport activity may restrict the actions of kinases, or other proteins involved in transporter regulation, by limiting the accessibility of regions of the protein important to modulation of function. In support of this hypothesis is the finding that both the PKC-mediated phosphorylation and internalization of the SERT are reduced in a dose-dependent manner by the presence of extracellular substrate (144). Furthermore, the prolonged presence of either substrate or inhibitor changes both the activity and surface

expression of the GAT1, independent of regulation by PKC (19). These findings suggest that changes in transporter conformation induced by active transport or inhibitor occupancy may alter both the normal trafficking and the PKC-mediated regulation of the transporters. However, we found no obvious difference in either the subcellular localization or PMA induced internalization of the GFP:DAT in the presence of extracellular substrate or inhibitor. However, minor changes in the localization or internalization of GFP:DAT due to the presence of substrate or inhibitor might be difficult to distinguish from experimental variation in our system. Furthermore, these reports are quite recent, and in the absence of confirming evidence, it remains possible the modulation of transporter trafficking and regulation by substrate may be limited a subset of to the Na^+/Cl^- transporter family.

The failure of extracellular substrate to alter GFP:DAT localization in the presence or absence of PMA may also represent limitations in the use of MDCK cells as a model system for examining regulation of transporter function. PC12 cells were used to demonstrate the substrate-mediated changes in PMA responsiveness of the endogenously expressed SERT. Although both PC12 and MDCK cells endogenously express Na^+/Cl^- transporters, as neuroendocrine cells, PC12 cells may express proteins important to transporter function and regulation that are not expressed by epithelial cells. However, it is important to note that the PC12 cells used to examine the substrate-mediated changes in SERT regulation were not induced to express a neuronal phenotype (144). Even more relevant perhaps, are the substrate-mediated changes in GAT1 distribution that were demonstrated in primary cultures of hippocampal neurons. Moreover, these results were reproducible in CHO cells, indicating that modulation of GAT1 activity by substrate is not confined to a particular cell type (19). However, GAT1 activity is not inhibited by PMA in CHO cells, suggesting that the compliment of proteins expressed by cells that are used in heterologous expression systems to examine

regulation of the transporters is of considerable importance, and must be considered in any conclusions drawn from the data obtained.

A direct comparison between the PMA-mediated inhibition of GFP:DAT activity and loss of GFP:DAT protein from the cell surface indicates that the rate of loss of function exceeds the rate of internalization (compare panels B and C of Fig. 4, manuscript 2). We observed a similar difference in loss of activity and decreased cell surface expression of the NET following application of PMA (data not shown). Furthermore, the rate of PMA-induced internalization of GFP:DAT observed by live-cell microscopy was slower than the rate of inhibition of transport activity. While the reduced rate of internalization in live cells may be due to the decreased temperature at which the assay was performed, there may be another explanation for the discrepancy in these findings. The majority of reports on regulation of the Na^+/Cl^- transporters by PKC conclude that inhibition of transport involves a decrease in V_{max} with no change in K_T. However, ours is the only study that either examines the rate of internalization or compares the rate of internalization to the rate of loss of function. The kinetic values from this and other studies are calculated from data determined at steady state following PMA treatment conditions that produce maximal effects. If therefore, uptake activity is being measured after internalization is complete or nearly complete, an accompanying change in KT might not be detected. This is especially true if the majority of the transporters at the cell surface at the time of assay were recently recruited to the plasma membrane and were not effected by PMA stimulation. Therefore, it is possible that activation of PKC has multiple effects on the transporters. The difference we observed between the rate of inhibition and the rate of internalization could be reconciled by alternative hypotheses. One possibility is that downregulation of the transporters results from two separate events both mediated by PKC; one producing a rapid decrease in affinity, followed or accompanied by a second that induces a slower internalization of the transporters.

Another possibility is that PKC inactivates the transporters, driving their internalization and degradation through a PKC-independent mechanism. This second possibility is supported by the finding that the GAT1 is internalized following incubation with an inhibitor in hippocampal neurons and CHO cells (19), suggesting that inactive transporters are removed from the cell surface. However, whether or not the GAT1 protein is degraded following inhibitor-mediated internalization is unknown. The question of whether a decrease in the affinity of the transporters for substrate precedes internalization can only be answered by performing saturation analysis at an early timepoint following treatment with a saturating concentration of PMA.

A report published in September, 1999 attempts to answer many of the same questions we addressed in this study, by examining the trafficking of the DAT both under basal conditions and following activation of PKC (101). While we relied on fluorescent detection methods to follow the localization of the transporter, Melikian and Buckley took a more biochemical approach, identifying the compartments containing the DAT by subcellular fractionation of postnuclear supernatants and immunoisolation of vesicles. The importance of selecting a suitable model system becomes apparent in comparing the data generated by these separate approaches. Melikian and Buckley expressed the hDAT in PC12 cells for their study of regulation and trafficking of the transporter. As discussed earlier, PC12 cells may provide a more relevant model system for studying molecules normally expressed in the CNS than do MDCK cells. Moreover, PC12 cells are an excellent model for studying the protein trafficking, as the identification of trafficking vesicles by fractionation is well established.

When stably expressed in either PC12 or MDCK cells, the DAT exhibits normal biochemical and functional characteristics (i.e. molecular weight, drug inhibition, kinetics). Furthermore, the effects of PMA on uptake activity are similar in both MDCK-GFP:DAT and DAT-PC12 cells. As is seen in many cell types, inhibition of the DAT in

PC12 cells is specific to the activation of PKC, and is associated with a decrease in the V_{max} of transport with no significant change in K_T. However, DAT activity is inhibited by PMA to a greater extent in PC12 cells than in MDCK cells. PMA-mediated stimulation of vesicular release mechanisms in PC12 cells could cause an apparent decrease in uptake activity by diluting the specific activity of radiolabeled dopamine. However, identical results were obtained in the presence of reserpine, an inhibitor of vesicular uptake, indicating that the release of dopamine did not effect the evaluation of DAT activity in PC12 cells.

Consistent with my results in MDCK cells, cell surface biotinylation indicates that DAT protein is lost from the surface of PC12 cells following incubation with PMA (101). Densitometric measurements of the immunoblots also revealed that in untreated cells more than 60% of the mature DAT is intracellular. This is in stark contrast to my findings using confocal microscopy, which indicate that under basal conditions, only a small proportion of GFP:DAT is intracellular. While the limited depth of optical slices obtained by confocal microscopy can be misleading in this regard, I have also found that the unbiotinylated fraction of untreated MDCK-GFP:DAT cells contains primarily the lower molecular weight immature form of the GFP:DAT protein, and only minor amounts of mature protein (data not shown). The predominance of immature GFP:DAT protein in the unbiotinylated fraction also suggests that the small amount of intracellular fluorescence seen in untreated GFP:DAT cells by confocal microscopy is due to the presence of immature protein in the ER.

Subcellular fractionation of DAT-PC12 cells was used to define the identity of vesicles containing the intracellular pool of DAT protein. Vesicles containing the DAT do not cofractionate with either the large dense-core vesicles or synaptic vesicles of PC12 cells, indicating that the high level of intracellular DAT does not result from accumulation of the protein in these vesicular structures, which have no counterpart in MDCK cells.

Additional fractionation shows an overlap in the migration of vesicles containing the DAT and vesicles containing the TfR, while vesicles containing the early endosomal markers EEA1 or rab5A fractionate at a somewhat lower density. However, the peak of DAT immunoreactivity was very broad, spanning twice as many fractions as the TfR, creating significant overlap with the early endosomal markers as well. Therefore the possibility the DAT is associated with multiple vesicle populations cannot be eliminated. However, since the majority of intracellular TfR is associated with endosomal recycling compartments (ERCs), the DAT-containing vesicles that comigrate with TfR containing vesicles are proposed to be ERCs. This is confirmed by immunoisolation of vesicles from the DAT/TfR containing fractions showing that vesicles containing both the DAT and TfR are precipitated with an α-TfR antibody.

Subcellular fractionation and vesicle immunoisolation were also used to show localization of the DAT after DAT-PC12 cells were incubated with PMA. To determine if the DAT from the cell surface was being internalized into ERCs following activation of PKC, surface proteins were biotin-labeled before application of PMA. After a 30 minute incubation with PMA, the remaining biotin was stripped from the cell surface with 2-mercaptoethanosulfonic acid (MesNa), and cell supernatants were fractionated. Biotin-labeled proteins were recovered from each fraction with streptavidin, and immunoblots were probed with a DAT-specific antibody. DAT immunoreactivity is observed in a distinct population of vesicles, and is significantly more abundant in the vesicles of PMA treated cells than in the vesicles of control cells. Vesicles from the peak DAT/TfR positive fractions of PMA treated cells were also immunoisloated with α-TfR antibody. As with immunoisolation of untreated cells, vesicles containing both the TfR and DAT were precipitated by the α-TfR antibody, but not by an irrelevant antibody. The authors conclude on the basis of the fractionation and immunoisolation results following PMA treatment that the DAT is internalized into ERCs following activation of PCK.

The results of the MesNa protection experiment provide some very important insights into the cellular trafficking of the DAT. While I was able to demonstrate by fluorescence microscopy that the population of transporters appearing in vesicles after activation of PKC came from the cell surface by first inhibiting protein synthesis, the ability to show protection of the biotin label from the reducing agent MesNa is certainly more definitive. Moreover, MesNa protection clearly shows that the DAT is undergoing endocytosis under basal conditions, as a small but significant amount of biotinylated DAT appears in the vesicular fraction. The appearance of biotinylated DAT in vesicles provides indirect evidence the DAT continuously cycles from a plasma membrane to an intracellular localization. However, the return of the MesNa protected DAT to the cell surface is not demonstrated. I attempted to perform protection assays under several conditions in MDCK cells, using either direct trypsin cleavage of surface transporters or reducing agents to release a biotin label. However, I was never able to demonstrate protection of the internalized transporters from either proteases or reducing agents. The success of this approach in PC12 cells suggests that the difficulties I encountered were a function of the cell type.

Based on the results of their study, Melikian and Buckley propose a model in which the DAT undergoes cycling between the plasma membrane and ERCs under basal conditions. While we have no direct evidence of DAT cycling in MDCK cells, there are some important distinctions between PC12 and MDCK cells. The most obvious, and perhaps most important being that while the majority of the DAT is intracellular in PC12 cells, the level of intracellular DAT appears to be much lower in MDCK cells.

The high percentage of intracellular DAT protein seen in PC12 cells poses several questions. What causes this apparent difference in localization between cell types? How might the differences in localization effect the results of experiments and the conclusions drawn? Does the localization accurately represent the localization of the DAT in vivo?

Melikian and Buckley screened several clones of DAT-PC12 cells, all of which exhibited a similar response to PMA in single point assays. However, whether they used a high-, low-, or moderate-expressing clone for their study is not known. In MDCK cells, the amount of intracellular protein appears to correlate to the level of protein expression. Therefore, increased intracellular levels of DAT in PC12 cells may be due to overexpression of the protein. However, if expression levels are excessively high in the DAT-PC12 cells, it would contradict the idea that high expression levels reduce the apparent inhibition of the transporters by increasing the repopulation of the cell surface from intracellular stores following internalization, since PMA-mediated inhibition is greater in DAT-PC12 cells than in GFP:DAT cells.

The difference in the ratio of intracellular to surface protein may also reflect a difference in protein trafficking between the two cell types. During internalization of the GFP:DAT colocalization with Tfn is transient and difficult to detect, suggesting that transit through early endosomes is occurring rapidly. Thus, cycling of the transporter in MDCK cells may occur more rapidly or with less frequency than in PC12 cells, increasing the ratio of transporter protein at the cell surface.

The high intracellular concentrations of DAT protein seen in PC12 cells may not be due to high expression levels, but may simply reflect a characteristic of transporter distribution in PC12 cells. Although PC12 cells may provide a better model in some regards than other cell types, including MDCK, the distribution of DAT in PC12 cells does not reflect what is seen *in vivo*. Immunogold labeling of dopaminergic terminals in the striatum showed that the DAT is almost exclusively associated with the plasma membrane (64).

Using a dominant negative mutant of dynamin1, we provide evidence that the DAT is internalized as a result of increased clathrin-mediated endocytosis following activation of PKC. The colocalization of the DAT and TfR in ERCs indirectly supports

the conclusion that internalization of the transporters occurs through CCPs. However, there is no direct evidence indicating that the carriers are clustered in CCPs under either basal conditions or following activation of PKC. Since recruitment of proteins to plasma membrane CCPs is mediated by the adaptor protein AP2, demonstrating an association between the carriers and AP2 by coimmunoprecipitation or yeast two-hybrid techniques would provide additional evidence that the transporters are localized to CCPs. Moreover, mutational analysis of intracellular domains will be needed to determine which residues of the transporters are required for AP2 binding and clathrin-mediated endocytosis.

Our finding that following activation of PKC the DAT is targeted to the lysosome for degradation appears to contradict a model of transporter recycling that is increased by PMA stimulation. However, while Melikian and Buckley state that the DAT was not being degraded, they only examined protein levels over the timecourse of their experiments. All of their assays were done immediately following a 30 minute incubation with PMA, and based on our results degradation occurs significantly more slowly. Therefore, degradation of the protein would not have been evident at the time DAT content was assessed. While the accumulation of DAT protein in ERCs suggests that it is not being targeted to the lysosome, Melikian and Buckley do not compare the density of DAT-containing vesicles to that of lysosomes. Furthermore, if the accumulation of DAT protein in intracellular compartments is due to a reduction in the rate of protein trafficking, the DAT may not accumulate in lysosomes to any appreciable degree within the times examined. Although there is no evidence for proteins being targeted to the lysosome from ERCs, the possibility cannot be eliminated. Accumulation of the DAT in ERCs following PKC activation suggests that it will return to the cell surface. A major flaw of Melikian and Buckley's model is that they provide no evidence that the DAT returns to the surface or that uptake activity is restored following PMA-mediated internalization.

The accumulation of the DAT in ERCs following PMA treatment suggests that the internalized transporter would return to the cell surface. However, my findings indicate that the internalization of the transporters in response to PMA is an irreversible process. The observation that PKC-mediated inhibition of the transporters is irreversible was also noted by other groups studying regulation of the DAT (38,189), however no data were provided to support these statements. On the other hand, there is no evidence that any of the transporters either regain function or return to the cell surface following PKC-mediated inhibition. The findings that inhibition of the transporters is irreversible came from experiments utilizing the phorbol ester PMA to stimulate PKC activity. Due to the hydrophobicity of PMA, a significant amount would be expected to remain associated with the cells following removal from the medium. Therefore, stimulation of PKC activity may not be terminated when PMA is removed. Although the more hydrophilic PKC activator phorbol-12,13-dibutyrate (PDBu) is more readily dissociated from cells, and has been used fairly extensively in the study of PKC-mediated regulation of the transporters, the effect of removal of PDBu is unknown.

Several of the findings from this study indicate that nearly the entire population of GFP:DAT molecules at the cell surface are internalized following activation of PKC. Data from cell surface biotinylation, fluorescence microscopy, protein degradation, and uptake assays all support a loss of at least 90% of the transporter from the surface of MDCK cells that have been pretreated with CHX. Although other studies have not examined regulation of the transporters in the absence of protein synthesis, many still report high levels of transporter inhibition in response to PMA. Melikian and Buckley, for example observed an 80% decrease in DAT activity in following activation of PKC (101). If, as I have proposed, repopulation of the plasma membrane under conditions where protein synthesis is not inhibited contributes in part to the remaining transport activity, inhibition would be even greater in the presence of protein synthesis inhibitors.

In neurons, protein synthesis and maturation occurs within the cell body, which is often far removed from the synaptic terminals where the transporter is localized. Therefore, synaptosomal preparations might be expected to more closely reflect conditions where protein synthesis is inhibited. However, while transport activity is decreased in response to phorbol esters in synaptosomes, the magnitude of inhibition, 20-40%, is rather modest (38,123,180) The relatively low level of PMA-mediated inhibition of the transporters observed in synaptosomal preparations suggests that although synaptosomes retain at least some of the proteins involved in regulation of the transporters, important components of protein trafficking may be lost.

Regulating the activity of a protein by targeting it for degradation would seem energetically unfavorable considering the amount of energy expended by the cell in protein synthesis. However there are many examples of the membrane proteins being internalized and degraded as a regulatory mechanism, particularly proteins involved in signaling. As discussed in the second manuscript of this thesis, several of the G-protein coupled receptors and the surface glycoprotein CD4 are downregulated by internalization and degradation in response to PKC activation. In addition to their regulation by PKC, many of the G-protein coupled receptors are also internalized when exposed to ligand, although ligand-mediated desensitization does not lead to lysosomal degradation. The internalization of other receptors, such as the TfR (157) and asialoglycoprotein receptor (158), in response to phorbol esters is associated with a decrease in the recycling rate. The accumulation of DAT in the ERCs suggests that regulation of the transporter by PMA in PC12 cells may be occurring by a similar manner. Therefore, the transporters may be subject to two different mechanisms of regulation, much like the G-protein coupled receptors. If so, then the pathway that produces a reduction in recycling rate might be dominant in PC12 cells, while the pathway that leads to degradation may dominate in MDCK cells. The number and variety of proteins that are targeted for

internalization by PKC suggests that a common cellular mechanism may be involved. However, the nature of such a signal remains unknown.

Desensitization or downregulation of receptors limits their activity in the presence of excess ligand. However, the function of the transporters as a mechanism of neurotransmitter clearance would be better served by an increase in activity in the presence of excess substrate. While the D_2 autoreceptor is generally believed to be inhibitory, there is evidence that D_2 receptor activation results in phosphoinositide release and calcium mobilization in some cell types (reviewed in 178). Activation of the D_2 autoreceptor by dopamine stimulates DAT activity (see introduction of this thesis), perhaps through the action of a calcium/calmodulin-dependent protein kinase. Although the activation of receptors other than the D_2 may be involved the inhibition of the transporters by PKC, there is evidence that the ability of PKC to downregulate transporter activity is restricted or may even be reversed by the presence of excess substrate (144). Therefore, the inhibitory effect of PKC activation on the transporters may be limited by the concentration of extracellular neurotransmitter.

While the activation of PKC has very profound effects on the Na^+/Cl^- transporters in heterologous expression systems, they may be more limited *in vivo*. PKC-mediated inhibition of the neurotransmitters may occur only at discreet locations within the cell. How the activation of a single kinase mediates a number of different cellular responses remains a fundamental question of biology. While there is a significant body of work addressing the subcellular localization of PKA in determining the specificity of its actions, little is known about how the actions of PKC isoforms are confined.

The response of neurotransmitter transporters to regulation by PKC may also be limited by the subcellular localization of the transporters themselves. While the association of neurotransmitter transporters with other proteins is an active area of research, little is yet known about the identity of such proteins. However, association of

the transporters with proteins that are restricted to particular regions of the cell may play a role in limiting the actions of PKC. A recent report demonstrated a direct interaction between the Na^+/Cl^- transporter BGT1 and the PDZ protein LIN-7 (127). PDZ proteins are involved in the localization of membrane proteins to specialized domains of neurons. Both the GluR1 glutamate receptor and the shaker potassium channel are retained in active zones through their interaction with PDZ proteins (163,191). However, the localization of the BGT1 in neurons, or the ability of other members of the Na^+Cl^- transporter family to associate with PDZ proteins remains unknown.

Internalization and degradation of a protein may be energetically favorable if a rapid and prolonged response is desired. Rapid and sustained changes in synaptic activity are a hallmark of longterm depression (LTD) and longterm potentiation (LTP); alterations in synaptic signaling that are associated with learning and memory. Furthermore, activation of PKC plays an important role in establishing LTP and LTD. The induction of LTP and LTD are accompanied by an increase or decrease, respectively, in the number of receptor molecules on the surface of postsynaptic neurons. Activation of PKC-coupled mGluRs or direct activation of PKC with PMA downregulates AMPA-type glutamate receptors in Purkinjie cells, facilitating the induction of LTP (156). Moreover, NMDA-type glutamate receptors are removed from the surface of hippocampal neurons in response to conditions that produce LTD (28). Initiation of LTP or LTD is limited to not only individual neurons, but to discreet synapses within individual neurons, suggesting that regulation of receptors channel is highly controlled. Therefore, while PKC activation results in the inhibition of the large majority of the Na^+/Cl^- transporters in model systems expressing the carriers, the actions of PKC may be much more limited *in vivo*. Although the role of presynaptic neurons in the induction of LTD and LTP is well established, the role of presynaptic neurons is less well understood. However, changes in the activity of postsynaptic receptors can also be achieved by

altering either the rate of release or the rate of reuptake in presynaptic neurons. Furthermore, while long-term changes in the dopamine system have been shown to be involved in learning, little is known about the development of LTP or LTD at dopaminergic synapses.

Long-term changes in dopaminergic neurotransmission are also associated with drug sensitization. In animal models, repeated exposure to psychostimulants, particularly cocaine, causes a potentiation in the response to subsequent psychostimulant administration. That is, psychostimulant "experienced" animals show a greater response to drug challenge than do naive animals, suggesting long-term alterations in the activity of catecholaminergic neurons due to exposure to the drug. Animals given repeated injections of cocaine show a marked reduction in dopamine uptake activity in the nucleus accumbens (30,71). Furthermore, mazindol binding sites are decreased in the nucleus accumbens following repeated cocaine administration, indicating a decrease in the number of dopamine transporters (159). While the signaling mechanisms involved in drug sensitization are unknown, administration of high doses of amphetamine causes an increase in the activity of PKC both in vitro and in vivo (48,49). Therefore, internalization and degradation of neurotransmitter transporters in response to activation of PKC may underlie the physiological alterations in transporter number and function observed during drug sensitization.

The results of this study define both the fate of neurotransmitter transporters following stimulation of PKC, and the cellular pathways involved. However, the intramolecular determinants of both normal and regulated trafficking of the neurotransmitter transporters have not been described. Sequences within the transporter molecules that are involved in cellular trafficking will be defined by identifying loss-of-function mutants. While the carboxyl terminus of the dopamine transporter does not appear to be involved in regulation by PKC (90), mutants in which other intracellular

domains of the GFP:DAT are truncated or deleted may provide insights into the location of regulated trafficking signals. Since the regulation of the TAUT by PKC has been attributed to a single amino acid residue (61) intracellular domains involved in regulation of GFP:DAT may also be identified by replacing intracellular loops of GFP:DAT with corresponding sequences of the TAUT that do not confer sensitivity to PKC. Replacement of the intracellular loop of the GFP:DAT between transmembrane domains 6 and 7 with the sequence of the TAUT containing the critical serine residue should provide a positive control for PKC-mediated regulation of GFP:DAT/TAUT chimeras.

The identification of cellular proteins involved in transporter trafficking and regulation also remains critical to our understanding of these processes. Using the yeast two-hybrid system to clone proteins that bind intracellular domains of the carriers will best identify proteins that interact with the transporters. Coimmunoprecipitation of cellular proteins will also provide information about cellular proteins that bind the transporters (e.i. molecular mass), and may be useful in identifying candidate proteins for further binding and localization studies. Having shown that the GFP:DAT is internalized through CCPs, it will be of some interest to determine whether wild type transporters or loss-of-function mutants can be captured by affinity chromatography on columns linked to adaptor proteins, such as AP-2, that are associated with clathrin lattices.

Although our understanding of how the activity of neurotransmitters is regulated in the brain remains limited, the results presented in this thesis provide some important insights into potential mechanisms of transporter regulation. Future studies will further define the role of PKC and other kinases in the regulation of the neurotransmitter transporters, and the relevance of transporter modulation to human health and behavior.

CONCLUSIONS

The goal of this study was to determine the role of protein kinases on the regulation of neurotransmitter transporter function. We find that while activation of PKA has no effect on transporter function, the activity of the dopamine transporter, GABA transporter, and norepinephrine transporter is inhibited by activation of PKC. Stimulation of PKC with phorbol esters decreases the maximal velocity of transport but has no effect on the affinity of the transporters for substrate. The activity of the transporters is inhibited by their rapid removal from the cell surface. Following activation of PKC, the transporters are translocated from the cell surface to intracellular compartments. The transporters are internalized through clathrin-coated pits into early endosomes. Internalized transporters transit through the endosomal-lysosomal pathway, and are targeted for degradation. Nearly all of the transporters found at the cell surface upon activation of PKC are degraded within 2 hours, and newly synthesized transporters repopulate the plasma membrane.

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Appendix

From

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[21] Selective Labeling of Neurotransmitter Transporters at the Cell Surface

By GWYNN M. DANIELS and SUSAN G. AMARA

Introduction

Neurotransmitter transporters, like all complex integral membrane proteins, undergo an extensive maturation process before becoming fully functional. During this maturation process, transporter molecules are found on the surface of the endoplasmic reticulum (ER), Golgi apparatus,

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and associated vesicles, as well as on the plasma membrane. Although the transporter protein half-life appears to be quite long (estimated at up to 6 days), under normal circumstances a significant amount of carrier protein will be found in subcellular compartments, rather than at the cell surface. This is especially true in transient transfection systems, where gene expression is maximized, and high concentrations of transporter protein can overwhelm the cellular maturation machinery. In this case, large amounts of immature protein may remain in the ER and Golgi, rather than being delivered to the plasma membrane. Therefore, selective labeling of only those molecules found at the surface of the cell may be desirable when examining a number of aspects related to transporter function.

Cell surface biotinylation serves as an important tool in several experimental approaches used to study neurotransmitter transport proteins, by identifying those molecules present only at the cell surface. This method is particularly useful for experiments that clarify changes in the functional properties of transporters, such as in the analysis of mechanisms of regulation of transport activity, or in the interpretation of results obtained from mutagenesis. Differences in the transport rate observed in these studies can have several plausible explanations, including alterations in biosynthesis, stability of the protein, catalytic activity, or the surface expression of the carrier. In structure–function studies, mutations become much more informative if it can be established that the carriers are efficiently targeted to and maintained at the cell surface, where they have the potential to be functional. Similarly, in experiments examining the modulation of transporter activity, the technique of cell-surface labeling offers a means to help distinguish whether changes in transport activity are due to recruitment or removal of carriers from the cell surface, or whether they reflect actual changes in the catalytic activity and/or substrate affinity of the transporter itself. Finally, the approach also offers the possibility of considering some of the biochemical properties of the transporters, such as the nature and extent of glycosylation or other posttranslational modifications, that are present on the plasma membrane.

The protocol presented below describes the use of membrane impermeant modified biotin molecules for the selective labeling of only those proteins present at the plasma membrane. With this approach, cell surface proteins are biotinylated with a hydrophilic, membrane-impermeant biotin derivative and affinity purified using an avidin resin; the carrier proteins are then identified using specific antibodies on immunoblots of biotinylated proteins.

Cell Surface Biotinylation

Introduction

Cell surface biotinylation is a powerful tool that is used to selectively label proteins found on the plasma membrane. It utilizes the high affinity of avidin for biotin ($K_d \sim 10^{-15}$) to separate integral membrane proteins from those residing in the cytoplasm or on the membranes of subcellular organelles. A protocol developed by Sargiacomo *et al.*[1] has been used extensively to examine the differential distribution of membrane proteins in polarized epithelial cells. Adaptations of this method were used to determine the localization of neurotransmitter transporters in MDCK cells,[2–4] as well as to assess the efficiency of delivery of the carriers to the cell surface.[5]

This technique utilizes a number of modifications of biotin, all of which are linked to an *N*-hydroxysuccinimide (NHS) ester. This highly reactive ester group forms stable amide bonds via nucleophilic attack of free amines. In intact cells, these membrane-impermeant molecules react with unblocked NH_2-terminal amino acid residues, or more commonly with the epsilon amine groups of lysine residues exposed at the cell surface. This procedure is therefore useful for studying membrane proteins, such as the neurotransmitter transporters, which are predicted to have extracellular loops containing multiple lysine residues. Sulfonation of the NHS group, which is soluble only in organic solvents, allows the biotinylation reagent to be dissolved in aqueous solutions. The addition of a long spacer arm between the NHS and biotin moieties increases the efficiency of avidin binding by reducing steric hindrance, resulting in greater access of the biotin to the biotin binding site on the avidin molecule. The sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin), used in the protocol described below, is also synthesized with a cleavable disulfide bond within the spacer arm. The biotin–avidin complex is readily released from proteins labeled with this reagent using concentrations of reducing agents normally associated with protein sample buffers.

[1] M. Sargiacomo, M. Lisanti, L. Graeve, A. Le Bivic, and E. Rodriguez-Boulan, *J. Membr. Biol.* **107,** 277 (1989).

[2] G. Pietrini, Y. J. Suh, L. Edelmann, G. Rudnick, and M. J. Caplan, *J. Biol. Chem.* **269,** 4668 (1994).

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[4] H. H. Gu, J. Ahn, M. J. Caplan, R. D. Blakely, A. I. Levey, and G. Rudnick, *J. Biol. Chem.* **271,** 18100 (1996).

[5] T. T. Nguyen and S. G. Amara, *J. Neurochem.* **67,** 645 (1996).

Cell Culture

Cell surface biotinylation has been used successfully to label plasma membrane proteins in a variety of cell types. They include polarized epithelial cells and cells grown in suspension, as well as many types of adherent cells. Cells are generally grown under standard culture conditions for the cell type being used. A variety of tissue culture dishes are suitable for this purpose, although it is recommended that they not be polylysine coated, as the free amine groups of the lysine will compete with the membrane proteins in the biotinylation reaction. The size of the plates or wells used is determined by the expression level of the protein of interest and the sensitivity of the detection assay. Therefore, the optimum surface area will need to be assessed for each protein examined.

Polarizing epithelial cells are used specifically to examine the targeting of plasma membrane proteins to the apical or basolateral surface of the cell (for a discussion of this, see Ref. 6). Madin–Darby canine kidney (MDCK), LLC-PK_1 (porcine kidney), and Caco-2 (human colon adenocarcinoma) cells are the cell lines most commonly used. Stably transfected cells are plated at high density on polycarbonate filter supports (Costar Transwell, Cambridge, MA) and grown for several days until they form a confluent monolayer. Cells grown in this manner will develop tight junctions, which separate the plasma membrane into distinct apical and basolateral domains. The tight junctions also create an effective barrier between the medium in the upper (apical) chamber of the well and that in the lower (basolateral) chamber, allowing the membrane proteins to be labeled exclusively on one surface or the other. The integrity of the tight junctions in the filter-grown cells is tested by one of two methods. Leakage across the monolayer is assessed by adding [^{3}H]inulin or [^{3}H]ouabain to the apical chamber. Following a 2-hr incubation, an aliquot of the medium from the lower chamber is counted by liquid scintillation spectrometry. Leakage across the monolayer is expressed as the percent of the total counts added to the apical chamber that leaked into the basolateral chamber. If leakage across the monolayer is greater than 1% of the total counts, the monolayer is not considered tight, and the cells should be discarded. Alternatively, the transepithelial electrical resistance can be measured using a Millicell-ERS apparatus (Millipore, Bedford, MA). The electrical resistance, which will vary from cell type to cell type, is measured in ohms (Ω) per cm^2 and increases dramatically when the cells form a tight monolayer.

Nonpolarizing cell types should be plated at moderate density and given adequate time in culture to recover from trypsinization and express the

[6] K. Matter and I. Mellman, *Curr. Op. Cell Biol.* **6**, 545 (1994).

membrane protein of interest. The nonpolarized delivery of membrane proteins to the cell surface of epithelial cells can also be assessed in cells that are grown on standard tissue culture plates rather than on filter supports.

Biotinylation Reaction

Reagents

PBS^{++}: Phosphate-buffered saline containing 0.1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$

NHS-SS-Biotin (Pierce, Rockford, IL)

Biotinylation Buffer: 2 m*M* $CaCl_2$, 150 m*M* NaCl, 10 m*M* triethanolamine, pH 7.5–9.0

Quench Buffer: 100 m*M* glycine in PBS^{++}

Procedure. All steps are carried out at 0–4° to reduce internalization of the biotin label. Growth medium is aspirated, and the cells washed three times for 10 min with ice-cold PBS^{++} to remove extraneous amines. Freshly prepared biotin solution (0.5–2.0 mg/ml) is added in a quantity sufficient to cover the cell layer. When labeling polarized epithelia, the surface of the cells not receiving NHS-SS-biotin is incubated in biotinylation buffer alone. Incubation in biotin solution is carried out for 20–25 min at 4° with gentle agitation. As hydrolysis of the NHS ester in aqueous solutions is the major competing reaction, repeating the incubation with fresh biotin solution may improve incorporation of the label.

Following the biotinylation reaction, unreacted NHS-SS-biotin is quenched by the addition of 100 m*M* glycine. The cells are rinsed twice with ice-cold quench buffer, followed by incubation in additional quench buffer at 4° for 20 min with gentle agitation. After quenching the cells are rinsed twice with ice-cold PBS^{++}.

Comments. The polarized distribution of membrane proteins can only be assessed accurately if the biotinylation reagent is confined to either the apical or the basolateral surface of the filter-grown epithelial cells. Therefore, when working with polarized epithelial cells, particular care should be taken not to disrupt the integrity of the monolayer during the aspiration and pipetting steps. In addition, buffers containing calcium are required to maintain epithelial tight junctions which disintegrate rapidly in the absence of free calcium. However, the presence of calcium is not necessary when working with nonpolarized cells.

As any free amino group will compete with surface proteins in the amide bond formation, it is important to avoid buffers containing free amines (e.g., Tris, glycine, azide). Interestingly, the replacement of phosphate buffer with 10 m*M* triethanolamine has been shown to increase the

efficiency of NHS-biotin incorporation.[7] Since *N*-hydroxysuccinimide reacts preferentially with amines in their unprotonated state, the efficiency of the biotinylation reaction may be improved by increasing the pH of the biotinylation buffer. Therefore, it is advisable to compare the results of experiments done over a range of pH values, from neutral to basic, to determine the pH for optimal labeling of a particular protein.

Both time and concentration dependence of the biotinylation reaction must also be experimentally determined. Concentrations of NHS-SS-biotin ranging from 0.5 to 2.0 mg/ml are standard. At higher concentrations it may be necessary to first dissolve the NHS-SS-biotin in room-temperature buffer and then immediately transfer the solution to ice.

Extensive washing with quench buffer ensures that any remaining NHS-SS-biotin is fully reacted before cell lysis, to avoid the possibility of labeling intracellular proteins.

Nonspecific binding of unlabeled proteins in the subsequent recovery procedure can be accounted for by including a control for each reaction condition in which the biotinylation buffer does not contain biotin.

Cell Lysis

Reagents

Lysis Buffer: 1% Triton X-100, 150 m*M* NaCl, 5 m*M* EDTA, 50 m*M* Tris, pH 7.5

Procedure. Cell monolayers grown on permeable filters are carefully excised from the support cups with a sharp scalpel. The cells are then scraped from the wells or filters into 1 ml lysis buffer. The cell lysate is triturated gently and transferred to a 1.6-ml microfuge tube. The membrane proteins are extracted on ice for 1 hr with occasional brief vortexing.

Following detergent extraction, the lysates are cleared by centrifuging at 14,000*g* for 10 min at 4°. Then, 900 μl of the supernatant is transferred to a new microfuge tube. Care is taken to avoid transferring any of the pelleted material.

Comments. In addition to forming polarized monolayers on filter membranes, epithelial cells grow in a monolayer on the side of the filter cup, often extending as high as the meniscus of the apical medium.[8] Because they are not growing on the filter, these cells do not form the strong tight junctions which exclude the biotinylation reagent from the basolateral surface when it is applied to the apical medium. Likewise, biotinylation solution which is applied to the basolateral surface does not reach this

[7] C. J. Gottardi and M. J. Caplan, *Science* **260,** 552 (1993).
[8] C. J. Gottardi, L. A. Dunbar, and M. J. Caplan, *Am. J. Physiol.* **268,** F285 (1995).

population of cells. Inclusion of these nonpolarized cells in the data analysis can lead to misinterpretation of the results; therefore, it is important to carefully separate them from the cells growing directly on the filter.

When comparing the surface distribution of proteins expressed in the same cell line under different conditions, it can be assumed that the number of cells per tissue culture dish will remain constant when the cells are plated at the same density. However, when comparing surface expression across cell lines, it will be impossible to maintain a consistent cell number. Therefore, it is necessary to normalize the data by assessing the total protein concentration. An aliquot of the cleared lysate should be assayed for protein concentration by BCA (Pierce), or another acceptable method, and cleared lysate containing an equivalent amount of protein carried through to the next step.

Recovery of Biotinylated Proteins

Reagents

UltraLink Immobilized NeutrAvidin (Pierce)

Lysis Buffer: 1% Triton X-100, 150 m*M* NaCl, 5 m*M* EDTA, 50 m*M* Tris, pH 7.5

High-Salt Wash Buffer: 0.1% Triton X-100, 500 m*M* NaCl, 5 m*M* EDTA, 50 m*M* Tris, pH 7.5

No-Salt Wash Buffer: 50 m*M* Tris, pH 7.5

Procedure. Biotin-labeled proteins are separated from unlabeled proteins by adding 50–200 μl NeutrAvidin resin (50% slurry) to the cleared lysate. The biotinylated proteins are allowed to bind to the NeutrAvidin resin by incubating at 4° with end-over-end mixing for 1–16 hr. The NeutrAvidin resin is pelleted by centrifuging at 5000*g* for 15 min at 4°. The supernatant is transferred to another microfuge tube and saved for further analysis. The pellet is then washed three times by vortexing briefly in 1 ml lysis buffer, after which the NeutrAvidin resin is repelleted by centrifuging at 5000*g* for 2 min at 4°. The pellet is subsequently washed twice in 1 ml high-salt wash buffer, and once in 1 ml no-salt wash buffer. After the no-salt wash buffer has been aspirated, the pellets are again centrifuged at 5000*g* for 2 min at 4° and as much residual buffer as possible is removed.

Comments. The NeutrAvidin pellet is quite loose, so care must be taken when aspirating to avoid losing any of the pelleted resin. It is better to leave behind a portion of the buffer than to risk losing part of the sample.

The volume of NeutrAvidin slurry used must be in excess of the number of binding sites present on the biotinylated proteins in the cleared lysate.

Failure to include sufficient NeutrAvidin resin will lead to incomplete biotin binding and a subsequent reduction in protein recovery.

The biotin–avidin binding reaction is quite rapid, and binding of the labeled proteins to the NeutrAvidin beads should be complete within 1 hr. However the binding step may proceed overnight to ensure that binding is complete.

Recovery of biotinylated proteins with UltraLink immobilized NeutrAvidin has a number of distinct advantages over the use of native avidin, or even streptavidin. Nonspecific protein binding is reduced significantly, improving the signal-to-noise ratio. The modification of charged amino acid residues found at the surface of the avidin molecule provides a more neutral isoelectric point, and the binding capacity has been increased more than 2-fold over that of NeutrAvidin-agarose by coupling the NeutrAvidin moiety to 3M Emphaze biosupport medium AB1.

The use of both high and low ionic strength washes should ensure that all nonbiotinylated proteins are removed from the NeutrAvidin resin, resulting in a preparation free from contaminating proteins.

Alternatively, biotin-labeled surface proteins can be immunoprecipitated with a specific antibody, separated by SDS–PAGE, transferred to a membrane support, and visualized using an antibiotin antibody. This method has the advantage of reducing contaminating proteins which may be nonspecifically labeled on a Western blot, but requires either the use of a nonhydrolyzable NHS-biotin conjugate or nonreducing conditions.

A two-step recovery method can also be utilized. With this technique the protein of interest is first purified by immunoprecipitation using a specific antiserum, and the biotinylated portion of the immunoprecipitated protein recovered by binding to the NeutrAvidin resin as described.

Electrophoresis and Immunoblotting

Reagents

2× SDS Sample Buffer containing 143 m*M* 2-mercaptoethanol
Immobilon-P transfer membrane (Millipore)
Blocking Buffer: 3% (w/v) bovine serum albumin (BSA), 2% (w/v) nonfat dry milk in PBS^{++}
Renaissance Western blot chemiluminescence reagent (NEN, Boston, MA)

Procedure. The biotinylated proteins are released from the pelleted NeutrAvidin resin by the addition of 40 μl of 2× SDS sample buffer. An aliquot of 20 μl of the supernatant (unbound fraction) from the NeutrAvidin pellet (see Recovery of Biotinylated Proteins) is added to 20 μl of 2× SDS

Discussion

Cell surface biotinylation has been useful in assessing the delivery of the norepinephrine transporter and its glycosylation mutants to the plasma membrane in both transiently and stably transfected cells.[5] Biotinylated transporters are strongly labeled in the biotin treated lanes, whereas no transporter protein is visible in cells which have been treated in an identical manner, but without the addition of NHS-SS-biotin (Fig. 1, A and D). In contrast, nonbiotinylated proteins (Fig. 1, B)[5] show roughly equivalent labeling in both the treated and untreated cells. The majority of the norepi-

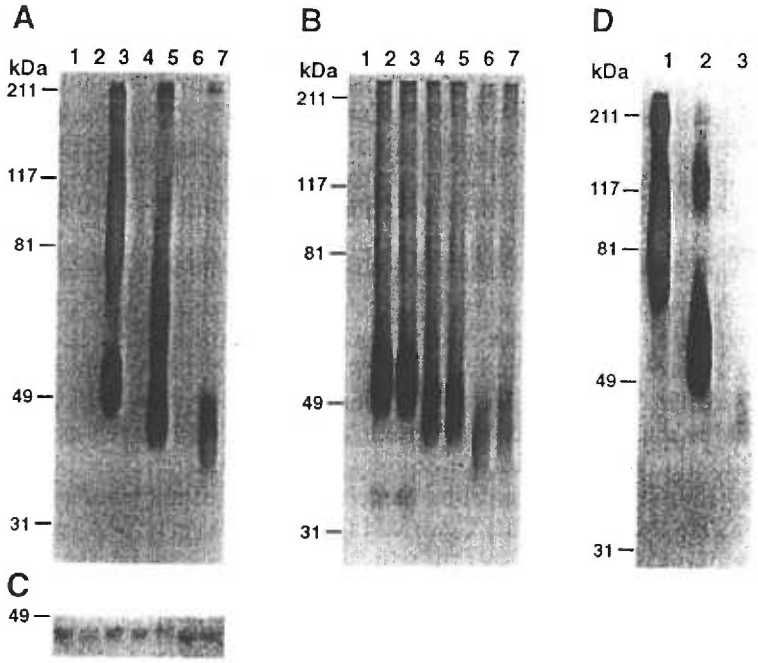

FIG. 1. Cell surface expression of the norepinephrine transporter (NET) and its glycosylation mutant proteins (NQQ and QQQ) in transiently transfected HeLa and stably transfected MDCK cells (A, B, and C) Control pBSK- (lane 1), NET (lanes 2 and 3), NQQ (lanes 4 and 5), or QQQ (lanes 6 and 7) transfected HeLa cells incubated in the presence (lanes 1, 3, 5, and 7) or absence (lanes 2, 4, and 6) of NHS-SS-biotin. (D) MDCK cells stably expressing NET (lane 1), NQQ (lane 2), or QQQ (lane 3). Biotinylated membrane proteins were isolated from 200 μg total protein with NeutrAvidin resin. Protein bound to the resin (A, C, and D) and 20 μg unbound protein (B) were separated by SDS–PAGE and transferred to membrane support. Western blots (A, B, and D) were probed with affinity purified α-NET antibody, followed by incubation with horseradish peroxidase-conjugated secondary antibody, and visualized by enhanced chemiluminescence. The blot from (A) was stripped and reprobed with α-actin monoclonal antibody (C). [Reproduced with permission from T. T. Nguyen and S. G. Amara, *J. Neurochem.* **67,** 645 (1996).]

nephrine transporter protein in the unbiotinylated fraction is found in the unglycosylated form, suggesting an intracellular location, whereas the fully glycosylated mature form of the protein is enriched at the cell surface.

Transfection systems can vary widely in the amount of protein produced, which can in turn result in marked differences in the interpretation of the data. Transient transfection systems, in general, produce large amounts of protein over a short period of time, while stable transfections yield lower protein levels at a steady state. Therefore, stable cell lines reflect expression levels more comparable to those found in the native state, and may provide a more accurate picture of what is happening *in situ*. The vaccinia virus : T7 expression system, in which cells are transfected with a gene under the control of the T7 promoter and infected with a recombinant vaccinia virus expressing the T7 polymerase, results in particularly high protein levels.[9] When the vaccinia virus : T7 expression system is used, large amounts of unglycosylated protein reach the cell surface (Fig. 1 A, lower band lanes 3, 5, and 7). Presumably this is because the secretory pathway of these cells is overwhelmed with transporter protein, allowing unglycosylated carriers, which are not normally delivered to the cell surface, to be sent to the plasma membrane. In contrast, cells which stably express the transporter have only the glycosylated form at the cell surface. The unbound fraction from NeutrAvidin isolation of surface proteins from these cells shows transporter primarily in the unglycosylated form (data not shown), further demonstrating that only the glycosylated form is directed to the cell surface.

To rule out the possibility that intracellular proteins were being biotinylated nonspecifically during the course of the biotinylation reaction, the blot from panel A was stripped and reprobed with an antibody directed against actin, a highly abundant cytoplasmic protein (Fig. 1, C). Only negligible amounts of actin were detected in the proteins bound to the NeutrAvidin beads, and the amount of actin observed was equivalent whether or not the cells were exposed to the biotinylation reagent. This result supports the idea that the actin detected reflects the nonspecific adsorption of this protein to the NeutrAvidin resin, and argues against the possibility that the biotinylation reagent gained access to intracellular proteins through cell lysis or leakage during the biotinylation reaction.

As the results of cell surface biotinylation experiments are qualitative, it is necessary to consider the efficiency of the labeling and recovery reactions when utilizing this procedure. Many conditions, such as the pH of the labeling buffer and the number of biotin binding sites available during NeutrAvidin recovery, can affect the outcome of the experiment. Incomplete labeling, nonsaturating NeutrAvidin binding, and loss of sample during wash steps can all result in low signal and misleading results. In addition,

[9] S. L. Povlock and S. G. Amara. *Methods Enzymol.* **296,** [29]. 1998 (this volume).

conditions which allow access of the biotin label to intracellular proteins can produce results which may be misinterpreted. However, cell surface biotinylation, when used judiciously, can provide important information on the localization of transporter protein.