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Regulation of the Functional Activity of the Human Dopamine Transporter by Protein Kinase C

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ABSTRACT. The role of protein kinase C (PKC) was examined in the regulation of dopamine transport in C6 glioma cells stably expressing the human dopamine transporter. The PKC activating phorbol esters phorbol 12-myristate 13-acetate (PMA) and 4β-12,13-dibutyrate phorbol-ester (PDBu) inhibited [^{3}H]dopamine uptake concentration dependently. These effects were attenuated by the PKC inhibitor staurosporine but were unaltered by another inhibitor, chelerythrine, or the phosphatase inhibitor okadaic acid. The potency of PMA in inhibiting [^{3}H]dopamine uptake was similar to that in inhibiting the binding of 2β-carbomethoxy-3β-(4-fluorophenyl)tropane ([^{3}H]WIN 35,428), and again staurosporine, but not chelerythrine, weakened the effect of PMA. The reduction in dopamine transporter activity by PMA was caused by a decrease in the V_{max} value of [^{3}H]dopamine uptake, opposed by a smaller reduction in the K_m value, whereas the effect of PMA on [^{3}H]WIN 35,428 binding was caused by a reduction in the B_{max} value without a change in the K_d value. The lower K_m value in the presence of PMA was accompanied by a higher IC_{50} of dopamine in inhibiting [^{3}H]WIN 35,428 binding; the latter effect was attenuated by the co-presence of staurosporine. The results are discussed in the context of transporter loss from the cell surface, or a model with phosphorylation affecting the shared dopamine and WIN 35,428 binding domain on the transporter as well as affecting a part of the dopamine binding domain lying outside that for WIN 35,428. BIOCHEM PHARMACOL 53;5:677–688, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. dopamine transporter; dopamine translocation; WIN 35,428 binding; protein kinase C; phosphorylation

Neurotransmitter transport plays an important role in dopamine transmission by clearing released neurotransmitter [1, 2]. Homozygous dopamine transporter knockout mice are spontaneously hyperactive and show extremely slow clearance of neuronally released dopamine [3], indicating the importance of the dopamine transporter in maintaining normal dopamine tone. The dopamine transporter is also a target in the action of psychostimulant drugs of abuse [4] and exogenous neurotoxins [5] potentially involved in the etiology of Parkinson's disease [6]. Renewed attention has been focused on dopamine transport in the case of schizophrenia [7, 8], which is generally believed to be associated with increased dopamine neurotransmission [9].

Evidence has been advanced in support of regulating Na^+, Cl^--dependent neurotransmitter transport by protein kinase-induced phosphorylation. Early on, White and Paton [10] suggested that ATP is required for phosphorylation of the norepinephrine carrier either before or after Na^+ binding, and Hendley and co-workers demonstrated a role for extracellular protein phosphorylation in the uptake of norepinephrine by synaptosomes [11] and PC 12 cells [12]. With regards to the serotonin transporter, there is evidence for the involvement of PKC,‡ with its activation being associated with a decrease in serotonin uptake by cultured endothelial cells [13] and platelets [14]. In contrast, increased serotonin uptake was observed upon activation of PKA, although this effect may be related to changes in transporter synthesis rather than regulation [15, 16]. Mixed effects on GABA uptake have been reported following agents that alter phosphorylation. Activation of PKC by phorbol esters in Xenopus oocytes expressing GAT1 resulted in an increase [17] or decrease [18] in GABA uptake, whereas in striatal synaptosomes a decrease [18] or no change [19] was observed. In the latter preparation, activation of PKA by forskolin and inhibition of protein phosphatases by okadaic acid reduced GABA uptake [19]. Little is known about regulation of dopamine transport by phosphorylation, although the cloned human and rat dopamine

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‡ *Abbreviations:* C6-hDAT, C6 glioma cell line expressing human dopamine transporter; GABA, γ-aminobutyric acid; PDBu, 4β-12,13-dibutyrate phorbol-ester; 4α-PDD, 4α-phorbol 12,13-didecanoate; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; 4α-PMA, 4α-phorbol 12-myristate 13-acetate; and WIN 35,428, 2β-carbomethoxy-3β-(4-fluorophenyl)tropane.

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transporters contain consensus sites for phosphorylation by PKA and PKC in the third intracellular loop and in the N- and C-terminal intracellular chains [20]. cAMP has been reported to enhance dopamine uptake into rat hypothalamic tuberoinfundibular dopamine neurons [21], and activation of PKC by PMA or 1-oleoyl-2-acetyl-*sn*-glycerol decreased dopamine uptake by COS cells expressing the cloned rat dopamine transporter [22]. All data taken together suggest the possibility that the function of the dopamine transporter is regulated by cyclicAMP and second messengers through protein kinase activity.

This study focuses on the role of PKC in the regulation of dopamine transport in C6-hDAT cells by monitoring the effects of PKC and phosphatase activators/inhibitors on the uptake of [^{3}H]dopamine and the binding of [^{3}H]WIN 35,428. [^{3}H]WIN 35,428 is a tropane analog of cocaine that binds with high affinity to the dopamine transporter complex [23–25], most likely involving a binding domain that, like in the case of cocaine, partially overlaps with the dopamine domain [26–30]. Both functional activity (dopamine uptake) and ligand binding (cocaine analog) were examined, and inhibition of binding by dopamine was measured as an indication of the affinity of dopamine for the carrier.

MATERIALS AND METHODS

Materials

Chemicals used in these experiments were obtained from the Sigma Chemical Co. (St. Louis, MO), except for [^{3}H]dopamine (37.54 Ci/mmol) and [^{3}H]WIN 35,428 (84.5 Ci/mmol) which were purchased from Dupont–New England Nuclear (Boston, MA), cocaine hydrochloride which was obtained from the Mallinckrodt Chemical Corp. (St. Louis, MO), and 4α-PDD which was from Research Biochemical Inc. (Natick, MA). All test compounds were dissolved in purified deionized H_2O prior to addition to buffers, except for chelerythrine, PMA, and PDBu, which were dissolved in dimethyl sulfoxide. The final concentrations of dimethyl sulfoxide in the assays were less than 0.5%, which by itself had no effect on [^{3}H]dopamine uptake or [^{3}H]WIN 35,428 binding.

C6-hDAT

hDAT cDNA was cloned in the laboratory of Dr. Aaron Janowsky (Oregon Health Sciences University, Portland, OR) by screening a human substantia nigra cDNA library with a polymerase chain reaction-amplified probe based on the rat cDNA sequence, and was stably transfected into rat C6 glioma cells, as described previously [31, 32]. Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 5% fetal bovine serum, 5% bovine calf serum, and 0.0001% puromycin (with pBabePuro conferring resistance to puromycin), and aliquots were frozen at a density of 10^7 cells/mL with 5% dimethyl sulfoxide. For each series of experiments, one freezing vial (1 mL) was thawed rapidly and seeded into a 75-cm^2 flask with the above medium. After 3 days of culturing ($\cong 3 \times 10^7$ cells total), the cells were collected by trypsinization and counted with a hemocytometer. Cells were then seeded into 96-well culture plates. Plates seeded at a density of 45,000 cells/well and 30,000 cells/well gave similar protein recoveries per well after 2 and 3 days of culturing, respectively.

Uptake of [^{3}H]Dopamine

The uptake assay was performed on cells grown on 96-well culture plates as described above. The culture medium was removed from each well in the plate. Cells were washed with 200 μL of wash buffer at room temperature (in millimolar concentrations: 122 NaCl, 5 KCl, 1.2 $MgSO_4$, 10 glucose, 1 $CaCl_2$, 15 Na_2HPO_4, and enough 0.85% H_3PO_4 to achieve a pH of 7.4). Subsequently, 70 μL of "uptake" buffer (wash buffer containing 10 μM nialamide) and 10 μL of water containing test drug were added. The cells were preincubated for 15 min at 21° with PKC activator/inhibitor, or phosphatase inhibitor, on a plate shaker, followed by the addition of 20 μL of assay buffer containing [^{3}H]dopamine (11 nM final concentration) and incubation for another 8 min. For studying varying concentrations of unlabeled dopamine, radiolabeled and unlabeled dopamine were added together and the incubation was continued for 3 min only. Uptake was terminated by putting the plate on ice and adding 100 μL of ice-cold wash buffer. The assay medium was removed immediately by aspiration, followed by two washes with 200 μL of ice-cold wash buffer. The cells in each well were incubated with 200 μL of 3% (w/v) ice-cold trichloroacetic acid for 30 min. The entire liquid content of each well was transferred to a scintillation vial and assayed for radioactivity with 5 mL of Cytoscint fluid (ICN, Costa Mesa, CA) by liquid scintillation counting in a Beckman model LS 6000 IC spectrometer at 50% efficiency in Cytoscint. Nonspecific uptake was defined with 100 μM cocaine. The cells in four separate wells were dissolved with 100 μL of 1 N NaOH, and the Folin phenol reagent method was used to determine the concentration of protein as described previously [33]. Approximately 20 μg C6-hDAT protein was obtained from one well.

To test for linearity of uptake with time, separate experiments were carried out with termination of assays at 1, 2, or 3 min. The zero-point was obtained by adding 100 μL of ice-cold wash buffer before adding 20 μL of [^{3}H]dopamine stock.

Binding of [^{3}H]WIN 35,428

Binding assays were carried out as the uptake measurements described above with the following changes. For binding assays with "uptake buffer" [^{3}H]WIN 35,428 was added (4 nM final concentration) instead of [^{3}H]dopamine. The incubation was continued on a plate shaker for 8 min at 21°. For binding assays with "binding buffer," this buffer (25

mM sodium phosphate buffer with 1 mM $CaCl_2$, brought to pH 7.4 by mixing 12.5 mM Na_2HPO_4 and 25 mM NaH_2PO_4, each containing 102 mM NaCl) was used throughout instead of assay buffer, and the binding assay was carried out for 8 min at 21°. The termination of the binding assays was identical to that of the uptake assays. In one set of experiments, this method was compared with dissolving cell material with 1% (w/v) sodium dodecyl sulfate; the two methods gave identical results. Nonspecific binding was defined with 1 μM WIN 35,428 to exclude the low-affinity portion of [^{3}H]WIN 35,428 found to be present in intact cell assays [34].

Data Analysis

One-way ANOVA was used to assess differences among treatment groups, with follow-up by the Least Significant Difference Multiple Range test for multiple comparisons or the Tukey Multiple Comparison test. ANOVA was also used to test for deviation from linearity in regression analysis. Where appropriate, paired or unpaired Student's *t*-tests were used. Deviations of pseudo-Hill numbers from unity and of inhibitions from 0% were assessed with the Student's one sample *t*-test. Differences were considered to be significant when $P \leq 0.05$. In some cases, data were log-transformed for homogeneity of variance. Saturation uptake data were analyzed with the nonlinear computer fitting program LIGAND [35] as described previously [33]. The IC_{50} values and pseudo-Hill numbers were computed with the equation of the ALLFIT program of De Lean *et al.* [36] entered into the Microsoft ORIGIN curve-fitting and plotting software. This nonlinear regression program was run with total and nonspecific uptake (binding) entered as constants.

RESULTS

Effect of PKC Activators/Inhibitors and a Phosphatase Inhibitor on [^{3}H]Dopamine Uptake

In the absence of test compounds, accumulation of [^{3}H]dopamine followed a linear pattern for the first 3 min, reached its peak value at 10 min, and subsequently decreased to a steady-state value at 30–40 min (Fig. 1A). When C6-hDAT cells were exposed to 2 μM PMA or PDBu, the time course of [^{3}H]dopamine uptake was suppressed persistently for up to 20 min as compared with the control time course (Fig. 1A). In separate experiments examining times of 0, 1, 2, and 3 min, the uptake was found to be linear in that time frame in both the absence and the presence of 2 μM PMA. [^{3}H]Dopamine uptake, allowed to occur for 8 min, was inhibited concentration-dependently by PMA or PDBu, both PKC-activating phorbol esters, with PMA being slightly more potent (Fig. 1B).

The effect of PMA on [^{3}H]dopamine uptake, measured for 8 min, was compared with that of three phorbol esters that are less active in stimulating PKC (Table 1). At 0.5 μM, 4α-PDD and 4α-PMA caused less inhibition (12–

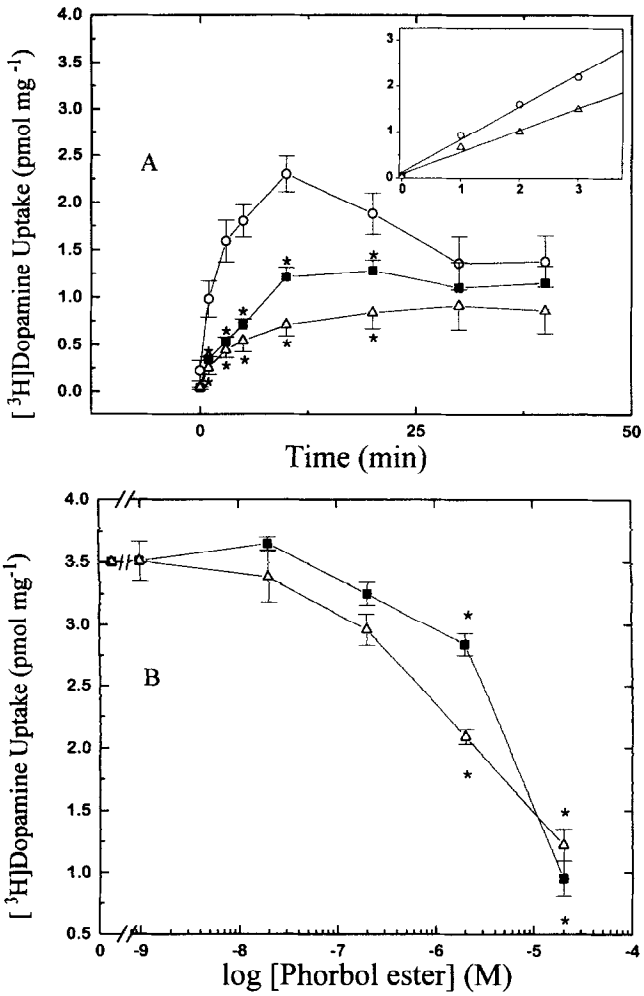

FIG. 1. Time course of [^{3}H]dopamine uptake and effect of PMA or PDBu. Cells were preincubated with or without a fixed (2 μM) (A) or varying (B) concentration of PMA or PDBu for 15 min at 21°, and [^{3}H]dopamine uptake was allowed to occur for the time indicated (A) or for 8 min (B). Key: (○) control; (■) PDBu; and (△) PMA. In panel A, results are means ± SEM for four independent cell preparations, each carried out in triplicate. In panel B, results are means ± SEM for three experiments, each in triplicate, with the same cell preparation, the entire experiment was replicated once with a different cell preparation. Inset panel A: Uptake of [^{3}H]dopamine was monitored for 1, 2, and 3 min. Results are means of three observations for a cell preparation different from those shown in the main panel. No significant deviation from linearity was observed for the control curve [$F(1,6) = 3.84$, $P = 0.11$] or PMA curve [$F(1,6) = 1.834$, $P = 0.22$]. Key: (*) $P < 0.05$ compared with absence of drug (one-way ANOVA followed by the Least Significant Difference Multiple Range test).

13%) than PMA (31%), whereas 4α-phorbol had no statistically significant inhibitory effect (9%). At 2 μM, the inhibitory effect of 4α-PDD was slightly greater than that of PMA (53 vs 40%), whereas 4α-PMA and 4α-phorbol had no statistically significant inhibitory effect (4–19%) (Table 1).

The PKC inhibitor staurosporine (0.3 μM) produced a slight reduction of 13% in [^{3}H]dopamine uptake measured

TABLE 1. Inhibition of [^{3}H]dopamine uptake into C6-hDAT cells by phorbol esters

Compound	% Inhibition at 0.5 μM	% Inhibition at 2μM
PMA	31 ± 6*[a] (11)	40 ± 3*[a] (19)
4α-PDD	12 ± 2*[b] (11)	53 ± 6*[b] (13)
4α-PMA	13 ± 3*[b] (4)	19 ± 7[c] (4)
4α-Phorbol	9 ± 7[b] (4)	4 ± 9[c] (4)

Values are mean ± SEM for the number of observations in triplicate indicated in parentheses. Percent inhibition is computed in reference to the control uptake measured in the absence of compound (3.7 pmol/mg protein). After a preincubation for 15 min at 21° with compound, [^{3}H]dopamine (11 nM final concentration) was added and uptake was allowed to occur for another 8 min.

* $P < 0.02$ compared with 0% (one sample t-test).

[a–c] $P < 0.05$ for comparisons between groups in the same column; groups labeled with different letters are statistically not from the same population, whereas groups with the same letters are (one-way ANOVA followed by the Least Significant Difference Multiple Range test).

for 8 min (Fig. 2A). When combined with 2 μM PMA or PDBu, which by themselves caused 34 or 25% inhibition of [^{3}H]dopamine uptake, the phorbol ester-induced inhibition was reversed and only the staurosporine effect itself, i.e. 13% inhibition, was observed (Fig. 2A). In contrast, concentrations of 0.02, 0.2, or 2 μM of the PKC inhibitor chelerythrine, which by themselves were ineffective, did not reverse the inhibition of [^{3}H]dopamine uptake by 2 μM PMA (Fig. 2B). Okadaic acid, a specific inhibitor of phosphatase 1 and 2A, at 0.3 μM, slightly reduced [^{3}H]dopamine uptake (Fig. 2C). This concentration of okadaic acid did not potentiate the inhibitory effect of either 2 μM PMA or PDBu. An additional set of experiments was carried out with a higher okadaic acid concentration of 2 μM, which by itself had no effect on uptake (3 ± 0.1% increase vs control, mean ± SEM of 3 observations in triplicate), 2 μM PMA (35 ± 1% decrease, $P < 0.001$ compared with

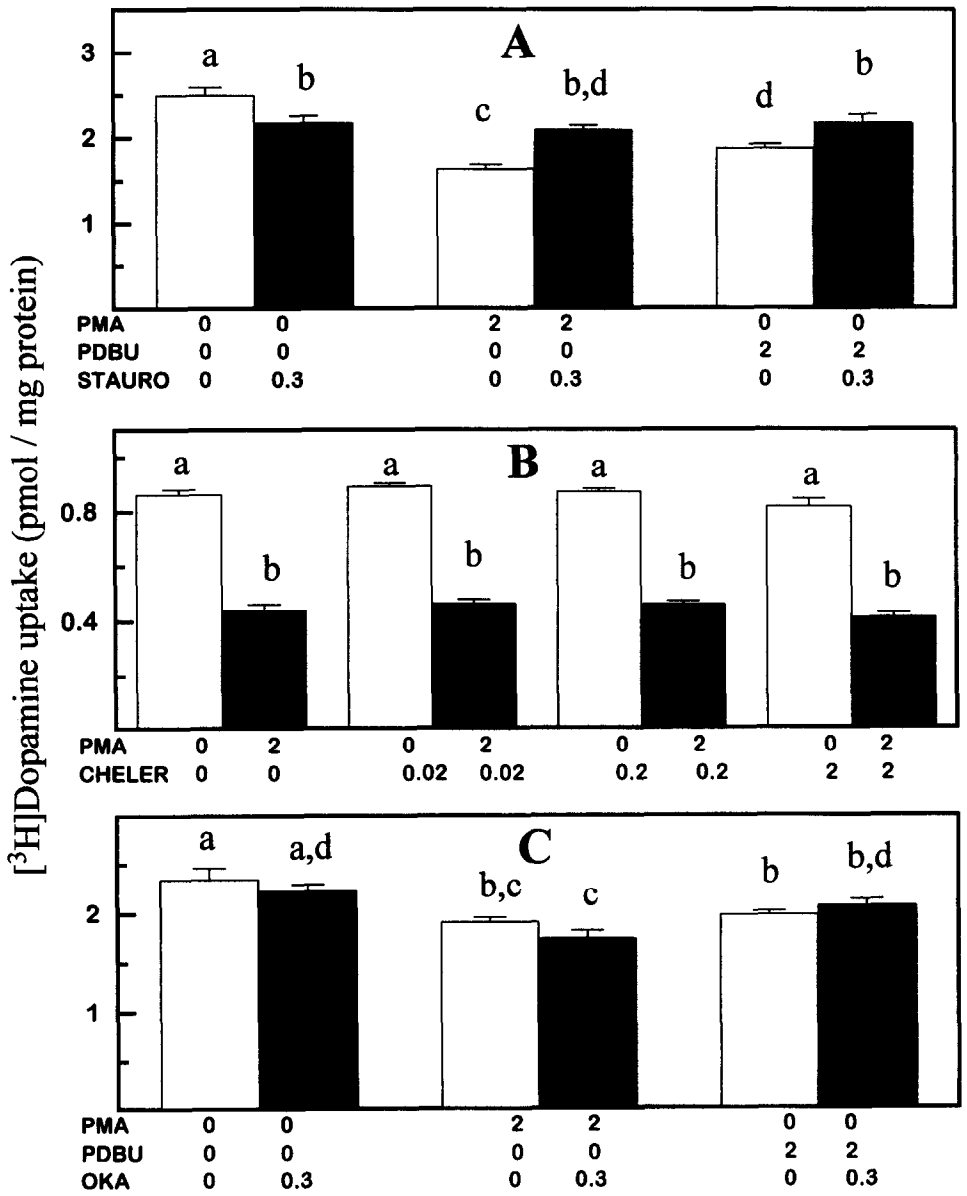

FIG. 2. Effect of staurosporine (STAURO) (A), chelerythrine (CHELER) (B), or okadaic acid (OKA) on phorbol ester-induced inhibition of [^{3}H]dopamine uptake. Cells were pretreated with phorbol ester or vehicle in the absence or presence of inhibitor at the micromolar concentrations indicated at 21° for 15 min prior to an 8-min exposure to [^{3}H]dopamine. Results are means ± SEM for three experiments, each in triplicate, with the same cell preparation. The entire experiment was replicated once with a different cell preparation. Key: (a–d) $P < 0.05$ for comparisons between groups; groups labeled with different letters are statistically not from the same population, whereas groups labeled with the same letters are (one-way ANOVA followed by the Least Significant Difference Multiple Range test).

control, Tukey Multiple Comparison test following significant one-way ANOVA), and the combination of PMA and okadaic acid (31 ± 1% decrease, $P < 0.001$ compared with control, $P > 0.05$ compared with PMA alone).

Effect of a PKC Activator on [^{3}H]Dopamine Uptake and [^{3}H]WIN 35,428 Binding

The concentration-dependency of inhibition by PMA was compared between [^{3}H]dopamine uptake and [^{3}H]WIN 35,428 binding under identical conditions ("uptake buffer") in parallel experiments carried out simultaneously (Fig. 3). Accumulation of [^{3}H]dopamine was monitored for 3 min in order to meet initial velocity conditions (Fig. 1A inset), whereas [^{3}H]WIN 35,428 binding was measured for 8 min, well within the equilibration plateau for the conditions used [34]. PMA inhibited [^{3}H]dopamine uptake with a potency (IC_{50} of 30 ± 4 μM) only slightly greater ($P < 0.01$) than that (IC_{50} of 48 ± 3 μM) for the inhibition of [^{3}H]WIN 35,428 binding (Fig. 3). There were no statistically significant differences between the Hill numbers for uptake (0.71 ± 0.10) and binding (0.82 ± 0.10) inhibition; the values taken together were lower than unity ($P < 0.01$).

Saturation analysis of [^{3}H]dopamine uptake (3 min) produced a monophasic Eadie–Hofstee plot (Fig. 4A). Treatment with PMA (2 μM) significantly decreased the V_{max} value from 108 ± 11 to 30 ± 4 pmol/mg protein/min ($P < 0.01$) and the K_m value from 2.2 ± 0.5 to 0.9 ± 0.04 μM ($P < 0.01$). Saturation analysis of [^{3}H]WIN 35,428 binding (with "binding buffer") produced a monophasic Scatchard plot (Fig. 4B). Treatment with PMA (2 μM) significantly decreased the B_{max} value from 1.7 ± 0.4 to 1.2 ± 0.3 pmol/mg protein ($P < 0.02$) but did not affect the K_d value, (10 ± 3 vs 9 ± 3 nM).

Effect of a PKC Activator and Inhibitor on the Affinity of Dopamine for [^{3}H]WIN 35,428 Binding Sites

In the absence of PMA, dopamine inhibited [^{3}H]WIN 35,428 binding, examined with the "uptake buffer," with a stronger potency (IC_{50} of 1.0 ± 0.1 μM) than in its presence (IC_{50} of 10.3 ± 3.6 μM) ($P < 0.01$) (Fig. 5A). In addition, the Hill numbers were appreciably reduced from 0.85 ± 0.03 to 0.27 ± 0.03 ($P < 0.0001$). Both groups of Hill numbers were significantly lower than unity ($P < 0.01$). The combination of staurosporine and PMA shifted the dopamine inhibition curve to the left as compared with PMA alone with a 10-fold reduction in IC_{50} ($P < 0.002$) and with an increase in Hill number ($P = 0.02$) (Fig. 5B). Again, the Hill numbers were lower than unity either with (0.42 ± 0.04) or without (0.27 ± 0.03) staurosporine ($P < 0.001$).

Effect of PKC Inhibitors on Inhibition of [^{3}H]WIN 35,428 Binding by a PKC Activator

In the following experiments, [^{3}H]WIN 35,428 binding, measured with the "binding buffer," was inhibited by varying concentrations of PMA in the presence and absence of a fixed concentration of staurosporine or chelerythrine. Staurosporine (0.3 μM) shifted the PMA inhibition curve significantly to the right with the IC_{50} going from 22 ± 9 to 76 ± 29 μM ($P < 0.05$) (Fig. 6A). In contrast, chelerythrine (2 μM) had no effect (Fig. 6B). The presence of kinase inhibitor did not affect the Hill numbers much (on the average 0.31 to 0.53 in the absence and 0.36 to 0.75 in the presence of inhibitor); except for the PMA and staurosporine combination, the Hill numbers were statistically sig-

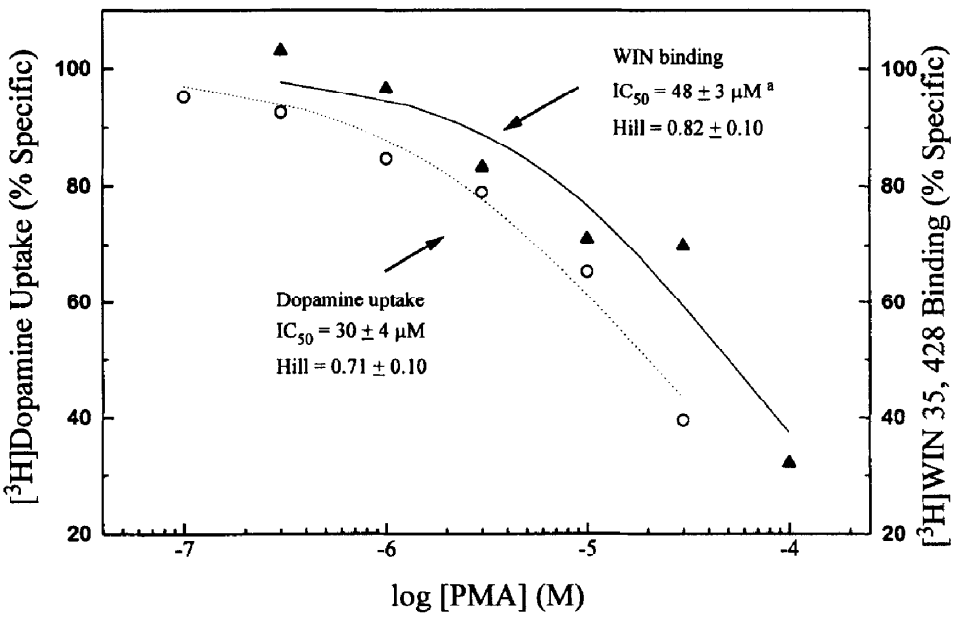

FIG. 3. Concentration-dependent inhibition of [^{3}H]dopamine uptake (○.....○) and [^{3}H]WIN 35,428 binding (▲—▲) by PMA. Cells were preincubated with various concentrations of PMA for 15 min at 21° prior to a 3-min exposure to [^{3}H]dopamine or an 8-min exposure to [^{3}H]WIN 35,428. Points shown are those obtained in a single experiment, assayed in triplicate, that was carried out four times. The insets show the means ± SEM of these results. The average control uptake in the absence of PMA was 1.71 pmol/mg of protein, and the corresponding binding was 0.28 pmol/mg of protein. Key: (a) $P < 0.01$ compared with corresponding measure for uptake (unpaired Student's *t*-test).

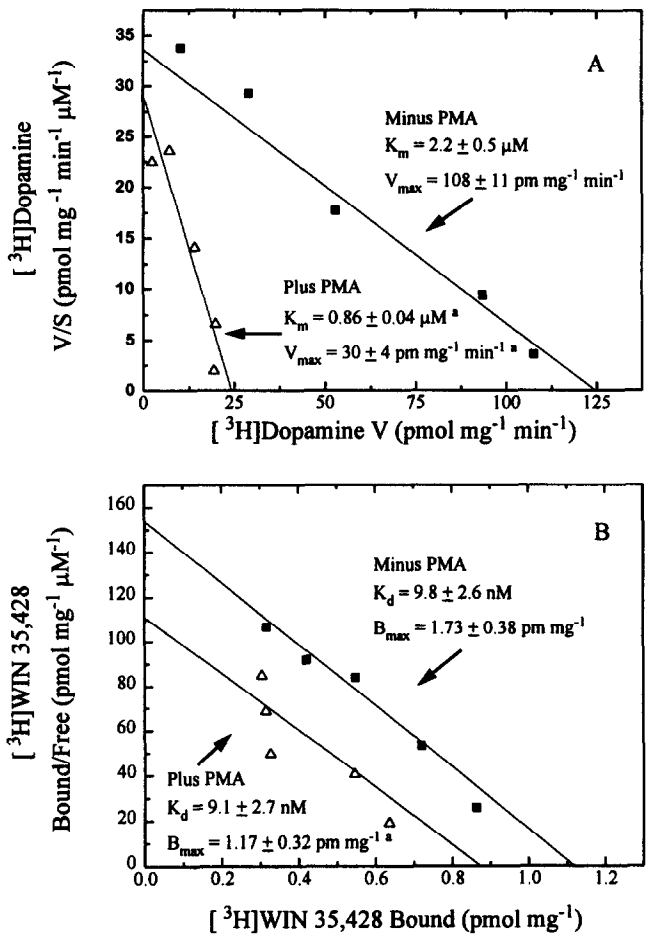

FIG. 4. Saturation analysis of [^{3}H]dopamine uptake (A) and of [^{3}H]WIN 35,428 binding (B) in the presence or absence of PMA (2 μM) added 15 min prior to the start of the assay. (A) [^{3}H]Dopamine was present at 11 nM and increasing concentrations of unlabeled dopamine (0.1, 0.3, 1, 3, and 10 μM) were added for a total assay time of 3 min. (B) [^{3}H]WIN 35,428 was present at 4 nM and increasing concentrations of unlabeled WIN 35,428 (1, 3, 10, 30, and 100 nM) were added for a total assay time of 8 min. The straight line represents the best fit chosen by the LIGAND program. Shown are typical experiments, assayed in triplicate, that were carried out six times. The insets show the means ± SEM of these results. Key: (■) no drug; (△) PMA; and (a) $P < 0.02$ compared with corresponding measure of the other group in the same panel (paired Student's *t*-test).

nificantly lower than unity ($P < 0.0001$). In the batch of C6-hDAT cells used for the experiments shown in Fig. 6B, PMA was somewhat more potent than generally observed in all other experiments.

DISCUSSION

Potency of PMA in Reducing Dopamine Transporter Function as Compared with Phorbol Esters That Are Less Active in Activating PKC

The micromolar concentrations of PMA required for inhibiting dopamine transporter function in the current system are considerably higher than the submicromolar concentrations employed in most studies including the report of Kitayama *et al.* [22] on the rat dopamine transporter expressed in COS cells. It is possible that the current C6 glioma cell expression system is less sensitive to phorbol esters perhaps by a more difficult redistribution of PKC into the membrane or a less favorable membrane environment for expression of activity of translocated PKC. The possibility was considered that micromolar concentrations of PMA caused nonspecific effects on [^{3}H]dopamine uptake unrelated to PKC activation. Although 4α-PDD did exert an inhibitory effect at 2 μM, 4α-PMA and 4α-phorbol had little or no effect at 2 μM. At the same concentration, PMA inhibited dopamine transporter function, and this was the concentration used in most experiments in this work. These results are consonant with the following possibilities. First, the effects of both PMA and 4α-PDD are unrelated to PKC activation. The present experiments do not allow us to definitely rule out this possibility. Second, the inhibition [^{3}H]dopamine uptake by 2 μM PMA is caused by PKC activation, and the effect of 4α-PDD needs an additional explanation. 4α-PDD may not be completely devoid of PKC stimulating activity, as micromolar levels of 4α-PDD have been reported to slightly inhibit serotonin uptake into pulmonary artery endothelial cells, a process sensitive to PKC activation [13]. Thus, it is possible that micromolar concentrations of 4α-PDD cause some PKC stimulation contributing to the inhibition of [^{3}H]dopamine uptake in the present experiments, although other mechanisms are likely to be involved in view of the appreciable inhibition observed by a 2 μM concentration of both PMA and 4α-PDD.

In this context, it should be pointed out that translocation of dopamine by the transporter is a complex process consisting of multiple steps [37] (see also below). Required ion gradients maintained by ion pumping membrane proteins may not be identical at different stages of the cycle of cell growth of the C6 glioma system used in the present experiments, even though the dopamine transporter protein is stably expressed in this transfected cell line. This may explain the observed variability in [^{3}H]dopamine uptake activity from experiment to experiment. The observed inter-experimental variation in the potency of PMA in inhibiting [^{3}H]dopamine uptake does not seem to be related to this phenomenon as there was no correlation between the potency of PMA in inhibiting uptake and the level of uptake ($r = 0.33$, $N = 7$, $P = 0.46$, % inhibition at 2 μM PMA vs control uptake in the absence of PMA, data of Figs. 1–4).

PKC Activation and Inhibition: Comparison with Published Observations

The reduction in the B_{max} value of [^{3}H]WIN 35,428 binding to C6-hDAT cells by PMA could be thought to result from a decrease in expression of dopamine transporter protein by the C6 glioma cells unrelated to PKC activation. However, this is unlikely if the dopamine transporter pro-

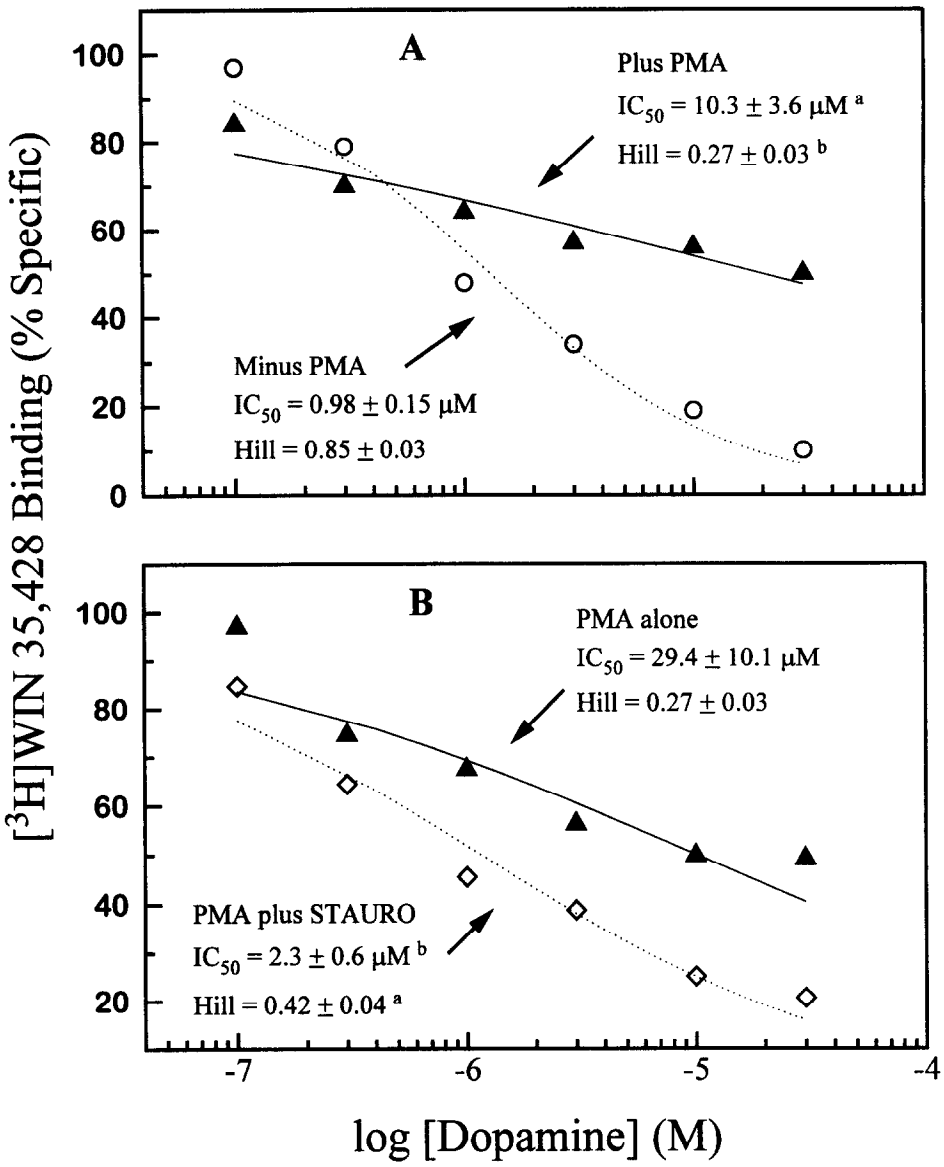

FIG. 5. Concentration-dependent inhibition of [^{3}H]WIN 35,428 binding by dopamine in the presence or absence of PMA (2 μM) or the combination of PMA (2 μM) and staurosporine (STAURO) (0.3 μM). Key: (○.....○) no drug; (▲——▲) PMA; and (◇.....◇) PMA plus STAURO. Cells were preincubated with PMA/STAURO for 15 min at 21° prior to an 8-min exposure to [^{3}H]WIN 35,428 and varying concentrations of dopamine. Data are shown as percent of specific binding in the absence of dopamine but in the presence of PMA or PMA/STAURO as applicable. Points shown are those obtained in a single experiment, assayed in triplicate, that was carried out five (A) or four (B) times. The insets show the means ± SEM of these results. The average control binding in the absence of PMA was 0.37 (A) or 0.52 (B) pmol/mg of protein; the corresponding value was 0.271 pmol/mg of protein with PMA (A), and 0.57 pmol/mg of protein with PMA plus STAURO (B). Key: (a) $P < 0.02$ compared with the corresponding measure of the other group in the same panel (unpaired Student's *t*-test); and (b) $P < 0.002$ compared with the corresponding measure of the other group in the same panel (unpaired Student's *t*-test).

tein in the current expression system turns over with a half-life comparable to that of 23 hr reported in the porcine kidney epithelial cell line LLC-PK$_1$ [38], which is appreciably longer than the time scale of 0.5 hr or less in the present experiments. Rather, the involvement of protein kinase activation in the effect of PMA on binding is indicated by its attenuation by staurosporine, a protein kinase inhibitor. The possibility exists that PMA induces loss of transporter from the cell surface, although examples of internalization have not been described for monoamine transporters. The counteractive effect of staurosporine on [^{3}H]dopamine uptake was observed in the present experiments with not only PMA but also PDBu. Although staurosporine is not very selective among the various protein kinases, PKC is the likely target because phorbol esters such as PMA and PDBu (non-metabolizable analogs of diacylglycerol) bind selectively to the regulatory domain of PKC to unmask the active site in the catalytic domain [39]. In this context, it is of interest that chelerythrine, a more specific PKC inhibitor, did not reverse the effect of PMA on [^{3}H]dopamine uptake at concentrations sufficiently high to inhibit PKC [40]. It should be kept in mind that the mechanism of PKC inhibition differs between staurosporine which acts at the catalytic domain without interacting with the ATP domain [39], and chelerythrine which targets probably both the substrate and ATP binding site within the catalytic domain [40], and that PKC, rather than being one enzyme, consists of a family of closely related enzymes [39]. It is feasible that the member of the PKC family that is involved in dopamine transporter regulation is not susceptible to chelerythrine. Differential sensitivity among PKC isoenzymes has been reported for the indolocarbazole inhibitor K252a which inhibits PKC-δ and -ε much more potently than -α, -β, -γ, and -ζ [41], the indolocarbazole inhibitor Go 6976 which selectively inhibits PKC-α and -β1 [42], and a staurosporine derivative which differentially affects PKC-α, -β, and -γ on the one hand and -δ on the other hand [43].

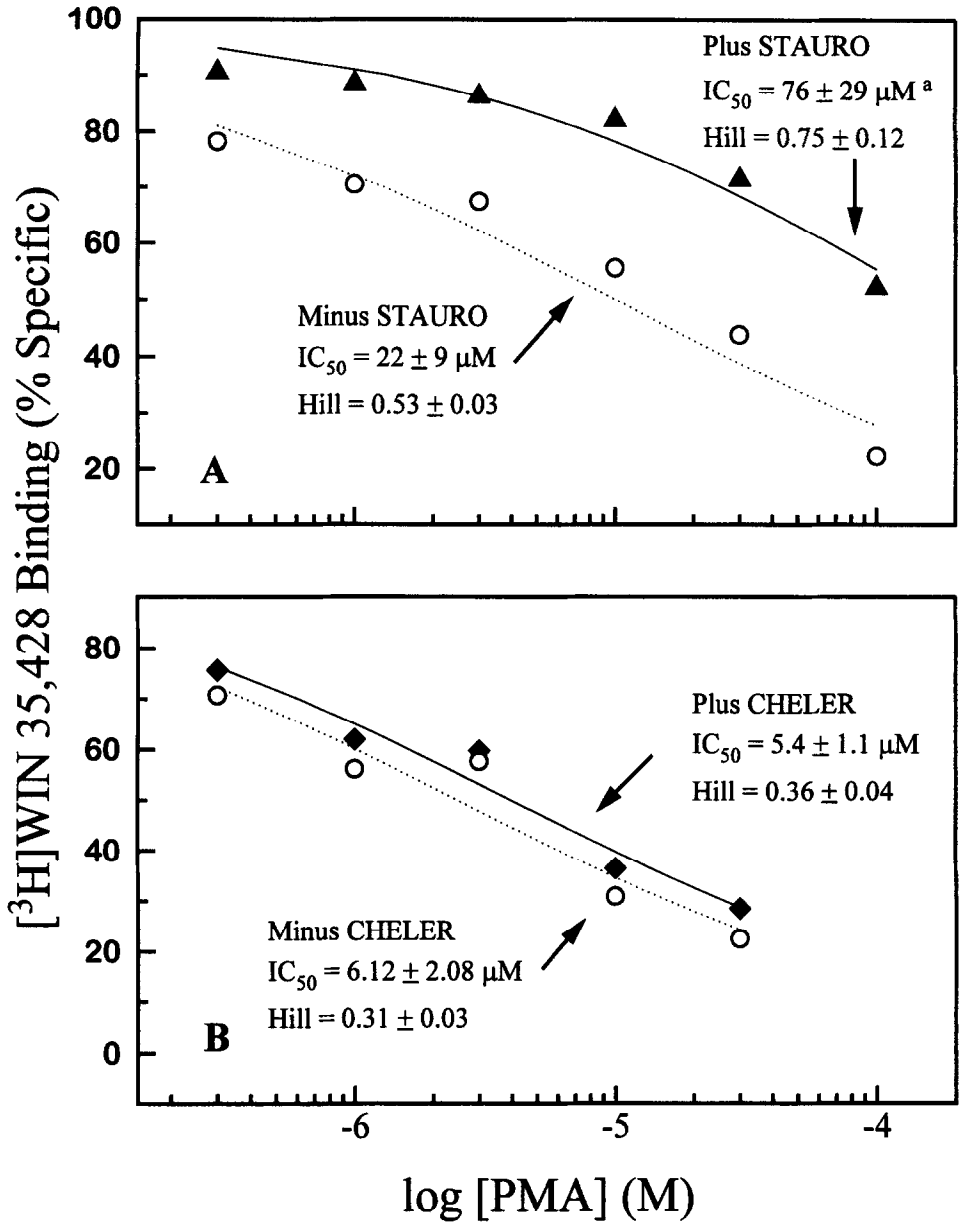

FIG. 6. Concentration-dependent inhibition of [^{3}H]WIN 35,428 binding by PMA in the presence or absence of staurosporine (STAURO) (0.3 μM) or chelerythrine (CHELER) (2 μM). Key: (○.....○) no drug; (▲——▲) STAURO; and (♦——♦) CHELER. Cells were preincubated with drug for 15 min at 21° prior to an 8-min exposure to [^{3}H]WIN 35,428 and varying concentrations of PMA. Data are shown as percent of specific binding in the absence of PMA but in the presence of STAURO or CHELER as applicable. Points shown are those obtained in a single experiment, assayed in triplicate, that was carried out six times. The insets show the means ± SEM of these results. The control binidng in the absence of drug was 0.25 (A) or 0.20 (B) pmol/mg of protein; the corresponding value was 0.27 pmol/mg of protein with STAURO (A), and 0.22 pmol/mg of protein with CHELER (B). Key: (a) $P < 0.05$ compared with the corresponding measure of the other group in the same panel (unpaired Student's *t*-test).

The present finding of a reduced V_{max} of [^{3}H]dopamine uptake with PMA agrees with previous findings reported for the rat dopamine transporter by Kitayama *et al.* [22]. However, the present reduction in the K_m value stands in contrast to the lack of effect reported in that study [22], perhaps reflecting a difference between the human and rat dopamine transporter, or a methodological difference such as the expression system used (C6 vs COS). Another difference between the two studies is the reduction in the B_{max} of [^{3}H]WIN 35,428 binding in the present experiments as opposed to the increase in the K_d value in the report of Kitayama *et al.* [22]. Regardless of the mechanism, the two studies share a phorbol ester-induced reduction in dopamine transporter function that is attenuated by staurosporine, suggestive of protein kinase involvement. The reduced V_{max} of [^{3}H]dopamine uptake by PMA would be in agreement with the possibility mentioned above consisting of loss of transporters from the cell surface by internalization.

Inhibition of Phosphatase: Comparison with Published Observations

Consonant with results reported for rat striatal synaptosomes [19], okadaic acid did not alter [^{3}H]dopamine uptake by C6-hDAT cells, nor did it enhance the effect of the phorbol esters PMA or PDBu in inhibiting dopamine uptake. The lack of effect of okadaic acid alone could be explained by postulating a threshold level of phosphorylated transporters (resulting from ongoing phosphorylating and dephosphorylating action) required for down-regulating dopamine uptake in conjuction with the postulated existence of spare transporters [33]; inhibition of only

a subset of protein serine/threonine phosphatases (PP-1, PP-2A [44]) by okadaic acid might not create a high enough level of phosphorylation as opposed to the effect of phorbol esters. The lack of potentiation of the dopamine uptake inhibitory effect of PMA and PDBu by okadaic acid, however, is harder to explain by this line of reasoning. Alternatively, dephosphorylation of the relevant residues in the dopamine transporter protein is carried out by a phosphatase that is less sensitive to okadaic acid, such as PP-2B (calcineurin) or not sensitive at all such as PP-2C [44].

Bugnon *et al.* [45] reported that okadaic acid induces a slight dopamine release from bovine retina and enhances K^+-, amphetamine-, or tyramine-induced dopamine release; all of these effects did not require extracellular Ca^{2+}. These findings were interpreted as supportive evidence for phosphatase 1 and/or 2A inhibiting transporter-dependent dopamine release, possibly through a direct effect on dopamine transporter function. Several possibilities exist. First, transporter function in the normal mode, taking up substrate from the extracellular to intracellular compartment, may be regulated by phosphorylation in a manner different from transporter function in the reversed mode. Second, it is possible that the bovine dopamine transporter is sufficiently different from that in the rat or human to explain opposite effects from an enhanced phosphorylation state (increased bovine but decreased rat or human dopamine transporter function). Third, the effects of okadaic acid in the bovine retina experiments may be more indirect; for example the authors themselves suggest an alternative possibility consisting of an increase in cytoplasmic dopamine [45].

Mechanism for Reduced Dopamine Transporter Function: Less likely Possibilities

Regulation of subcellular redistribution of transporters is an intriguing potential mechanism for modulating dopamine clearance. Such a mechanism has been shown to be susceptible to PKC for the rat brain GABA (GAT1) transporter [17]. However, the time scale for changes of this type is much longer (days) [17] than that of the present experiments (<0.5 hr) which is more like the time frame of the glucose transporter relocation in response to insulin [46]. Another possibility is that changes in intracellular ionic composition underlie the effect of PKC activation of dopamine transport. In fact, glial cells are known to be endowed with the Na^+/H^+ exchanger [47], the Na^+/Ca^{2+} exchanger [48], the $Na^+/HCO3^-$ co-transporter [49], and Na^+, K^+-ATPase [50], all enzymes involved in maintaining the transmembrane ion gradients; phosphorylation of at least one of these enzymes, the Na^+/H^+ exchanger, is likely to occur [51]. In addition, phosphorylation of glial non-gated K^+ channels, which determine the high K^+ permeability underlying the glial membrane potential [52], could impact on dopamine transport because the latter is known to be electrogenic [37]. However, all these explanations would require us to view the effects of PMA (with or without staurosporine) on dopamine transport as being entirely distinct from those on [^{3}H]WIN 35,428 binding. Yet, there is overwhelming evidence that dopamine and WIN 35,428 interact with common binding domains on the transporter, in addition to the involvement of separate domains [26–30]. Furthermore, [^{3}H]WIN 35,428 is likely to bind predominantly on the external face of the cell membrane where [Na^+] is high and [K^+] low [53]. The parallel effects on dopamine uptake and WIN 35,428 binding may therefore be related, obviating the need to invoke phosphorylation phenomena that only apply to dopamine transport. In this context it is interesting that the potency of PMA in reducing [^{3}H]dopamine uptake was quite similar to that in inhibiting [^{3}H]WIN 35,428 binding when the two assays were performed under identical conditions, and that staurosporine counteracted the effect of phorbol esters both on [^{3}H]dopamine uptake and on [^{3}H]WIN 35,428 binding.

Mechanism for Reduced Dopamine Transporter Function: More likely Possibilities

Although there is no agreement on the exact location of the substrate and blocker recognition domains from recent mutagenesis [54, 55] and chimera [56, 57] studies, the likely candidates so far are located within the transmembrane domains, whereas the PKC consensus sites are in the large intracellular N-terminal chain and in the small intracellular loop between the 4th and 5th transmembrane domain [20]. Thus, the phosphorylation sites may lie outside the substrate or blocker domains with phosphorylation involving conformational changes affecting dopamine or WIN 35,428 recognition. However, it should be kept in mind that a consensus sequence is neither sufficient nor necessary for the phosphorylating action of a protein kinase [44]. Placement of phosphorylation sites within dopamine/WIN 35,428 domains is contemplated in the following speculative model. Activation of PKC by phorbol esters reduces the number of available, functionally active dopamine transporters, causing a decrease in the V_{max} of [^{3}H]dopamine uptake. In addition, phosphorylation creates transporter protein that is unable to bind [^{3}H]WIN 35,428, causing a reduction in the B_{max} value. The remaining functionally active transporters bind [^{3}H]WIN 35,428 with unchanged affinity (same K_d) but take up [^{3}H]dopamine with a reduced K_m (see below). If we accept a shared domain involved in the binding of [^{3}H]WIN 35,428 and dopamine [26–30], then the lower potency of dopamine in inhibiting [^{3}H]WIN 35,428 binding after PMA treatment indicates a lower affinity of dopamine for the transporter (see below for the apparently contradictory *reduction* in the K_m of [^{3}H]dopamine uptake). This could be the result of phosphorylation of residues lying inside the dopamine binding domain, but outside the WIN 35,428 binding domain because these phosphorylated transporters still recognize [^{3}H]WIN 35,428 with the same K_d. A phosphorylation effect appears to be involved in the change in the affinity of

dopamine upon PMA tratment because the co-presence of staurosporine prevents the reduction in affinity and tends to normalize the affinity of dopamine towards that observed in the absence of PMA. The present results suggest two types of concurrent phosphorylation effects. The first type involves sites in the dopamine domain but outside the WIN 35,428 binding domain which, when phosphorylated, affect dopamine but not WIN 35,428 binding. The second type affects both the dopamine and WIN 35,428 domain causing loss of not only [^{3}H]dopamine uptake but also [^{3}H]WIN 35,428 binding; the responsible phosphorylation targets could be in the domain that is shared by dopamine and WIN 35,428. The affinity of dopamine for transporters phosphorylated by the second type of mechanism is unknown because these transporters do not bind [^{3}H]WIN 35,428, but we speculate it is low. Of course it should be emphasized that the above model is hypothetical, and that phosphorylation of the transporter is yet to be documented.

Factors Affecting both K_m and V_{max} of [^{3}H]Dopamine Uptake

If [^{3}H]WIN 35,428 labels the dopamine transporters remaining functionally active in taking up [^{3}H]dopamine after PMA treatment, it remains to be explained why the uptake K_m is reduced at the same time that the affinity of dopamine, as measured by inhibition of [^{3}H]WIN 35,428 binding, is *reduced*. In this context, it is important to recall that the K_m is a complex parameter consisting of many more components than the corresponding K_d parameter measured in binding experiments [58]. A good example of this can be found in the model outlined by Schomig *et al.* [59] involving binding, translocation, release, and reorientation rate constants. Using their definitions and equations, in the absence of inhibitor, one can derive that $K_m = K_S\ (1 + k_2/k_{-1})\ (k_3/[k_3 + k_2])$ and $V_{max} = C_{TOT} \cdot k_2\ (k_3/[k_3 + k_2])$, in which K_S = equilibrium dissociation constant for external substrate binding, C_{TOT} = total concentration of carrier, k_{-1} = dissociation rate constant for external substrate binding, k_2 = rate constant for translocation of loaded carrier and internal substrate release, and k_3 = reorientation rate constant. Thus, a change in k_3 would affect both K_m and V_{max} equally because it occurs only in the term that is shared between the two parameters, $(k_3/[k_3 + k_2])$. It is possible that PMA reduces the reorientation rate constant, which will lower $(k_3/[k_3 + k_2])$ thus exerting a lowering effect on both K_m and V_{max}. In considering the effect of PMA on the K_m, the reduction in k_3 by type 1 phosphorylation (see above) must be greater than the increase in K_S predicted from the decrease in dopamine affinity observed in the [^{3}H]WIN 35,428 binding experiments. For the effect of PMA on the V_{max}, the type 2 phosphorylation can be thought to reduce the number of available functional carriers, causing a decrease in C_{TOT}, whereas the type 1 phosphorylation reduces k_3. The combination of both of these effects may explain the greater effect of PMA on the V_{max} than the B_{max}. With regard to substrate recognition, it is of interest that PMA not only renders dopamine weaker in inhibiting [^{3}H]WIN 35,428 binding, but also makes the dopamine inhibition curve more flat with a lower Hill number as compared with the absence of PMA or with the combination of PMA and staurosporine. This may reflect a different mechanism of interaction between dopamine and the phosphorylated transporter, perhaps involving conformational changes.

Concluding remarks

The present results and those of Kitayama *et al.* [22] stand in contrast to the suggestion of Giambalvo [60] that PKC activation enhances dopamine transporter function and that PKC inhibition reduces function. The latter conclusion was based on a complex design of amphetamine-induced dopamine release from synaptosomes in the presence of PKC activators or inhibitors, and measurement of amphetamine-induced changes in PKC activity in different subcellular fractions. Clearly, the effects of amphetamine and its derivatives are complex [61] and may involve PKC-sensitive steps distal from the dopamine transporter. Be that as it may, regulation of dopamine transport by PKC is a potentially important mechanism for modulating dopamine transmission. In addition to the action of the classic second messenger diacylglycerol on PKC, other membrane phospholipids take part in activating PKC such as products originating in the phospholipase A_2-induced phosphatidylcholine hydrolysis pathway [62]. Thus, different pathways can converge upon PKC to regulate dopamine transmission through changes in dopamine transporter function [63].

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References

1. Uhl GR and Johnson PS, Neurotransmitter transporters: Three important gene families for neuronal function. *J Exp Biol* **196:** 229–236, 1994.
2. Garris PA, Ciolkowski EL, Pastore P and Wightman RM, Efflux of dopamine from the synaptic cleft in the nucleus accumbens of the rat brain. *J Neurosci* **14:** 6084–6093, 1994.
3. Giros B, Jaber M, Jones SR, Wightman RM and Caron MG, Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* **379:** 606–612, 1996.
4. Ritz MC, Lamb RJ, Goldberg SR and Kuhar MJ, Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science* **237:** 1219–1223, 1987.
5. Pifl C, Giros B and Caron MG, Dopamine transporter expression confers cytotoxicity to low doses of the parkinsonism-inducing neurotoxin 1-methyl-4-phenylpyridinium. *J Neurosci* **13:** 4246–4253, 1993.
6. Uhl GR, Walther D, Mash D, Faucheux B and Javoy-Agid F, Dopamine transporter messenger RNA in Parkinson's disease and control substantia nigra neurons. *Ann Neurol* **35:** 494–498, 1994.
7. Bannon MJ, Granneman JG and Kapatos G, The dopamine transporter. Potential involvement in neuropsychiatric disorders. In: *Psychopharmacology: The Fourth Generation of Progress* (Eds. Bloom FE and Kupfer DJ), pp. 179–188. Raven Press, New York, 1995.

8. Daniels J, Williams J, Asherson P, McGuffin P and Owen M, No association between schizophrenia and polymorphisms within the genes for debrisoquine 4-hydroxylase (CYP2D6) and the dopamine transporter (DAT). *Am J Med Genet* **60:** 85–87, 1995.
9. Seeman P and Niznik HB, Dopamine receptors and transporters in Parkinson's disease and schizophrenia. *FASEB J* **4:** 2737–2744, 1990.
10. White TD and Paton DM, Effects of external Na^+ and K^+ on the initial rates of noradrenaline uptake by synaptosomes prepared from rat brain. *Biochim Biophys Acta* **266:** 116–127, 1972.
11. Hendley ED, Whittemore SR, Chaffee JE and Ehrlich YH, Regulation of norepinephrine uptake by adenine nucleotides and divalent cations: Role for extracellular protein phosphorylation. *J Neurochem* **50:** 263–273, 1988.
12. Hardwick JC, Ehrlich YH and Hendley ED, Extracellular ATP stimulates norepinephrine uptake in PC12 cells, *J Neurochem* **53:** 1512–1518, 1989.
13. Myers CL, Lazo JS and Pitt BR, Translocation of protein kinase C is associated with inhibition of 5-HT uptake by cultured endothelial cells. *Am J Physiol* **257:** L253–L258, 1989.
14. Anderson GM and Horne WC, Activators of protein kinase C decrease serotonin transport in human platelets. *Biochim Biophys Acta* **1137:** 331–337, 1992.
15. Cool DR, Leibach FH, Bhalla VK, Mahesh VB and Ganapathy V, Expression and cyclic AMP-dependent regulation of a high affinity serotonin transporter in the human placental choriocarcinoma cell line (JAR). *J Biol Chem* **266:** 15750–15757, 1991.
16. Ramamoorthy S, Cool DR, Mahesh VB, Leibach FH, Melikian HE, Blakely RD and Ganapathy V, Regulation of the human serotonin transporter. Cholera toxin-induced stimulation of serotonin uptake in human placental choriocarcinoma cells is accompanied by increased serotonin transporter mRNA levels and serotonin transporter-specific ligand binding. *J Biol Chem* **268:** 21626–21631, 1993.
17. Corey JL, Davidson N, Lester HA, Brecha N and Quick MW, Protein kinase C modulates the activity of a cloned γ-aminobutyric acid transporter expressed in *Xenopus* oocytes via regulated subcellular redistribution of the transporter. *J Biol Chem* **269:** 14759–14767, 1994.
18. Osawa I, Saito N, Koga T and Tanaka C, Phorbol ester-induced inhibition of GABA uptake by synaptosomes and by Xenopus oocytes expressing GABA transporter (GAT1). *Neurosci Res* **19:** 287–293, 1994.
19. Tian Y, Kapatos G, Granneman JG and Bannon MJ, Dopamine and gamma-aminobutyric acid transporters: Differential regulation by agents that promote phosphorylation. *Neurosci Lett* **173:** 143–146, 1994.
20. Giros B and Caron MG, Molecular characterization of the dopamine transporter. *Trends Pharmacol Sci* **14:** 43–49, 1993.
21. Kadowaki K, Hirota K, Koike K, Ohmichi M, Kiyama H, Miyake A and Tanizawa O, Adenosine 3′,5′-cyclic monophosphate enhances dopamine accumulation in rat hypothalamic cell culture containing dopaminergic neurons. *Neuroendocrinology* **52:** 256–261, 1990.
22. Kitayama S, Dohi T and Uhl GR, Phorbol esters alter functions of the expressed dopamine transporter. *Eur J Pharmacol* **268:** 115–119, 1994.
23. Boja JW, Cline EJ, Carroll FI, Lewin AH, Philip A, Dannals R, Wong D, Scheffel U and Kuhar MJ, High potency cocaine analogs: Neurochemical, imaging, and behavioral studies. *Ann NY Acad Sci* **654:** 282–291, 1992.
24. Madras BK, Spealman RD, Fahey MA, Neumeyer JL, Saha JK and Milius RA, Cocaine receptors labeled by [^{3}H]2β-carbomethoxy-3β-(4-fluorophenyl)tropane. *Mol Pharmacol* **36:** 518–524, 1989.
25. Coffey LL and Reith MEA, [^{3}H]WIN 35,428 binding to the dopamine uptake carrier. I. Effect of tonicity and buffer composition. *J Neurosci Methods* **51:** 23–30, 1994.
26. Reith MEA, de Costa B, Rice KC and Jacobson AE, Evidence for mutually exclusive binding of cocaine, BTCP, GBR 12935, and dopamine to the dopamine transporter. *Eur J Pharmacol* **227:** 417–425, 1992.
27. Johnson KM, Bergmann JS and Kozikowski AP, Cocaine and dopamine differentially protect [^{3}H]mazindol binding sites from alkylation by *N*-ethylmaleimide. *Eur J Pharmacol* **227:** 411–415, 1992.
28. Saadouni S, Refahi-Lyamani F, Costentin J and Bonnet JJ, Cocaine and GBR 12783 recognize nonidentical, overlapping binding domains on the dopamine neuronal carrier. *Eur J Pharmacol* **268:** 187–197, 1994.
29. Reith MEA and Selmeci G, Radiolabeling of dopamine uptake sites in mouse striatum: Comparison of binding sites for cocaine, mazindol, and GBR 12935. *Naunyn Schmiedebergs Arch Pharmacol* **345:** 309–318, 1992.
30. Pristupa ZB, Wilson JM, Hoffman BJ, Kish SJ and Niznik HB, Pharmacological heterogeneity of the cloned and native human dopamine transporter: Disassociation of [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 binding. *Mol Pharmacol* **45:** 125–135, 1994.
31. Eshleman AJ, Neve RL, Janowsky A and Neve KA, Characterization of a recombinant human dopamine transporter in multiple cell lines. *J Pharmacol Exp Ther* **274:** 276–283, 1995.
32. Eshleman AJ, Henningsen RA, Neve KA and Janowsky A, Release of dopamine via the human transporter. *Mol Pharmacol* **45:** 312–316, 1994.
33. Xu C, Coffey LL and Reith MEA, Translocation of dopamine and binding of 2β-carbomethoxy-3 β-(4-fluorophenyl) tropane (WIN 35,428) measured under identical conditions in rat striatal synaptosomal preparations. Inhibition by various blockers. *Biochem Pharmacol* **49:** 339–350, 1995.
34. Reith MEA, Xu C, Zhang L and Coffey LL, Translocation of dopamine and binding of WIN 35,428 measured under identical conditions in cells expressing the cloned human dopamine transporter. *Naunyn Schmiedebergs Arch Pharmacol* **354:** 295–304, 1996.
35. Munson PJ and Rodbard D, LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107:** 220–239, 1980.
36. De Lean A, Munson PJ and Rodbard D, Simultaneous analysis of families of sigmoidal curves: Application to bioassay, radioligand assay, and physiological dose–response curves. *Am J Physiol* **235:** E97–E102, 1978.
37. Rudnick G and Clark J, From synapse to vesicle: The reuptake and storage of biogenic amine neurotransmitters. *Biochim Biophys Acta* **1144:** 249–263, 1993.
38. Mantel J-C, Cerruti C, Fleckenstein A, Carroll FI, Kuhar MJ and Patel AP, Turnover of rat dopamine transporter protein in rDAT-LLC-PK1 cells: Use of an irreversible ligand (RTI-76) and antisense oligonucleotides. *Soc Neurosci Abstr* **21:** 376, 1996.
39. Huang KP, The mechanism of protein kinase C activation. *Trends Neurosci* **12:** 425–432, 1989.
40. Herbert JM, Augereau JM, Gleye J and Maffrand JP, Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* **172:** 993–999, 1990.
41. Gschwendt M, Leibersperger H, Kittstein W and Marks F, Protein kinase Cζ and η in murine epidermis. TPA induces down-regulation of PKCη but not PKCζ. *FEBS Lett* **307:** 151–155, 1992.
42. Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marme D and Schachtele C, Selective

inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J Biol Chem* **268:** 9194–9197, 1993.
43. Gschwendt M, Furstenberger G, Leibersperger H, Kittstein W, Lindner D, Rudolph C, Barth H, Kleinschroth J, Marme D and Schachtele C, Lack of an effect of novel inhibitors with high specificity for protein kinase C on the action of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate on mouse skin *in vivo*. *Carcinogenesis* **16:** 107–111, 1995.
44. Girault J-A, Protein kinases and phosphatases. *Neurotransmissions* **10:** 1–6, 1996.
45. Bugnon O, Ofori S and Schorderet M, Okadaic acid modulates exocytotic and transporter-dependent release of dopamine in bovine retina *in vitro*. *Naunyn Schmiedebergs Arch Pharmacol* **351:** 53–59, 1995.
46. Simpson IA and Cushman SW, Hormonal regulation of mammalian glucose transport. *Annu Rev Biochem* **55:** 1059–1089, 1986.
47. Pappas CA and Ransom BR, Depolarization-induced alkalinization (DIA) in rat hippocampal astrocytes. *J Neurophysiol* **72:** 2816–2826, 1994.
48. Koller H, Buchholz J and Siebler M, Bacterial endotoxins impair electrophysiological properties of cultured astrocytes but not of cultured neurons. *J Neurol Sci* **124:** 156–162, 1994.
49. Deitmer JW and Schlue WR, An inwardly directed electrogenic sodium-bicarbonate co-transport in leech glial cells. *J Physiol (Lond)* **411:** 179–194, 1989.
50. Dolapchieva S, Ultracytochemical localization of ouabain-sensitive K^+-dependent paranitrophenylphosphatase in rat sciatic nerve fibers. *Eur J Morphol* **33:** 247–255, 1995.
51. Vigne P, Frelin C and Lazdunski M, The Na^+/H^+ antiport is activated by serum and phorbol esters in proliferating myoblasts but not in differentiated myotubes. Properties of the activation process. *J Biol Chem* **260:** 8008–8013, 1985.
52. Koester J, Membrane potential. In: *Principles of Neural Science* (Eds. Kandel ER, Schwartz JH and Jessel TM), pp. 81–94. Elsevier, New York, 1996.
53. Reith MEA and Coffey LL, Cationic and anionic requirements for the binding of 2β-carbomethoxy-3β-(4-fluorophenyl)[^{3}H]tropane to the dopamine uptake carrier. *J Neurochem* **61:** 167–177, 1993.
54. Kitayama S, Shimada S, Xu H, Markham L, Donovan DM and Uhl GR, Dopamine transporter site-directed mutations differentially alter substrate transport and cocaine binding. *Proc Natl Acad Sci USA* **89:** 7782–7785, 1992.
55. Kitayama S, Shimada S, Donovan D and Uhl G, Dopamine transporter residues important for substrate transport and ligand recognition. *Soc Neurosci Abstr* **18:** 1331, 1992.
56. Giros B, Wang YM, Suter S, McLeskey SB, Pifl C and Caron MG, Delineation of discrete domains for substrate, cocaine, and tricyclic antidepressant interactions using chimeric dopamine-norepinephrine transporters. *J Biol Chem* **269:** 15985–15988, 1994.
57. Buck KJ and Amara SG, Chimeric dopamine-norepinephrine transporters delineate structural domains influencing selectivity for catecholamines and 1-methyl-4-phenylpyridinium. *Proc Natl Acad Sci USA* **91:** 12584–12588, 1994.
58. Christensen NH, *Biological Transport*. W. A. Benjamin, Inc., London, 1975.
59. Schömig E, Michael-Hepp J and Schönfeld C-L, Inhibition of neuronal noradrenaline transport (uptake$_1$) and desipramine binding by amiloride and ethylisopropylamiloride. *Naunyn Schmiedebergs Arch Pharmacol* **340:** 495–501, 1989.
60. Giambalvo CT, Protein kinase C and dopamine transport—2. Effects of amphetamine *in vitro*. *Neuropharmacology* **31:** 1211–1222, 1992.
61. Schuldiner S, Steiner-Mordoch S, Yelin R, Wall SC and Rudnick G, Amphetamine derivatives interact with both plasma membrane and secretory vesicle biogenic amine transporters. *Mol Pharmacol* **44:** 1227–1231, 1993.
62. Asaoka Y, Nakamura S, Yoshida K and Nishizuka Y, Protein kinase C, calcium and phospholipid degradation. *Trends Biochem Sci* **17:** 414–417, 1992.
63. Zhang L and Reith MEA, Regulation of the functional activity of the human dopamine transporter by the arachidonic acid pathway. *Eur J Pharmacol* **315:** 345–354, 1996.