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Modulation of dopamine transporter functions by protein kinase C

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Abbreviations: DAT, dopamine transporter; PMA, phorbol 12-myristrate 13-acetate; MPP^+, 1-methyl-4-phenylpyridinium; PKC, protein kinase C; PKA, cAMP-dependent protein kinase A; CaM kinase II, Ca^{2+}-calmodulin-dependent kinase II; DMEM, Dulbecco's modified Eagle medium; CFT, 2β-carbomethoxy-3β-(4-fluorophenyl) tropane; CNS, central nervous system

Abstract

The effects of phosphorylation on the functions of the bovine dopamine transporter (DAT) expressed in CV-1 cells were investigated. The cells expressing DAT stably were prepared by transfection of CV-1 cells (monkey kidney cell line) with plasmid pRc/CMV-DAT which is an expression vector pRc/CMV-based plasmid containing bovine DAT cDNA (Usdin *et al.*, 1991). DAT functional assays followed the preincubations of DAT expressing cells with various modulators of protein kinases, such as phorbol 12-myristrate 13-acetate (PMA), staurosphorin, calphostin C, forskolin, and EGTA, on the concentration- or time-dependent manners. Dopamine uptake into DAT expressing cells was severely decreased by PMA pretreatment, and increased by staurosphorin or calphostin C. Similar effects were observed on the uptakes of 1-methyl-4-phenylpyridinium (MPP^+) into DAT-expressing cells. However, DAT functions were not altered by forskolin or EGTA. These results suggest that the functions of DAT are modulated by protein kinase C (PKC).

Keywords: dopamine transporter, modulation, phosphorylation, protein kinase C

Introduction

Dopamine transporter (DAT) is a highly specialized membrane-spanning protein that aids in terminating dopaminergic neurotransmission by sodium-dependent reuptake of dopamine into presynaptic neurons (Iversen, 1971; Horn, 1990; Shimada *et al.*, 1991; Uhl, 1992; Uhl and Hartig, 1992). This transporter also acts as a gate for dopaminergic neurotoxins such as 1-methyl-4-phenylpyridinium (MPP^+) (Snyder and D'Amato; 1986, Uhl, 1990; Kitayama *et al.*, 1992a), and the recognition sites for various psychotropic drugs including cocaine (Shimada *et al.*, 1991; Usdin *et al.*, 1991).

All the species homologues of the DAT (rat, bovine, and human) possess consensus sites for phosphorylation by cAMP-dependent protein kinase A (PKA), Ca^{2+}-calmodulin-dependent kinase II (CaM kinase II) and protein kinase C (PKC). The existence of putative phosphorylation sites indicates that the functions of DAT may be regulated by protein kinases or phosphatases.

The involvements of PKC and PKA in down-regulation of receptors for neurotransmitters have been well established, and several data have also suggested the possibilities that the functions of neurotransmitter transporters are regulated by protein kinases. The 5-HT transporter activity of human placental choriocarcinoma cells has been shown to be modulated by various protein kinase activators (Cool *et al.*, 1991). It has been suggested that glutamate transporter activity is increased in cultured glial cells after incubation of the cells with phorbol esters (Casado *et al.*, 1991), and this stimulation of activity by phorbol esters is due to a direct phosphorylation of the transporter by PKC (Casado *et al.*, 1993). The involvement of PKA in gamma-aminobutyric acid (GABA) transporter regulation has been also suggested (Tian *et al.*, 1994). Here, we demonstrate the possible implications of phosphorylation by PKC in the regulation of DAT actions.

Materials and Methods

Preparation of DAT-expressing cell line

To obtain the cells, which stably express DAT and in which the phosphorylation effects on the functions of DAT could be studied, CV-1 cells (monkey kidney cell line) were transfected with plasmid pRc/CMV-DAT which is an expression vector pRc/CMV-based plasmid containing bovine DAT cDNA (Usdin *et al.*, 1991). The transfection was carried out using liposome (Felgner *et al.*, 1987). The Lipofectin reagent (10 μl, GIBCO BRL,

Gaitherburg, MA) and DNA (2 μg) were mixed to form complexes. These DNA-Lipofectin complexes were added on CV-1 cells (1-2 x 10^5 cells) plated in 60-mm tissue culture plate and allowed to be introduced into cells for 24 h. After then, transfectants were selected by growing cells in G418 (geneticin, 400 μg/ml, Gibco BRL, Gaitherburg, MA)-containing medium. Most of the transfectant colonies expressed the functional DAT as seen by acquisition of functional dopamine uptake activity. The cells showing the highest dopamine uptake activity were used in the following experiments.

Cell culture

The CV-1 cells were cultured at 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. The culture conditions of pRc/CMV-DAT -transfectants were identical to those of CV-1 cells, except that 250 μg/ml of G418 were supplemented to the medium.

Functional analyses of DAT

1-2 x 10^5 DAT-expressing cells were plated in 24-well plate. After 24 h, the cells were washed with uptake buffer (25 mM Hepes, pH 7.4/125 mM NaCl/4.8 mM KCl/1.2 mM KH_2PO_4/1.3 mM $CaCl_2$/1.2 mM $MgSO_4$/5.6 mM glucose/1 mM sodium ascorbate/10 μM pargyline), incubated in the uptake buffer for 10 min at 37˚C and then treated with 100 nM [^{3}H]dopamine (51 Ci/mmol, Amersham, England) or 10 nM [^{3}H]MPP$^+$ (83 Ci/mmol, Dupont-New England Nuclear, Boston, MA) for 10 min at 37˚C. The uptake reactions were terminated by adding ice-cold uptake buffer and three washes with ice-cold uptake buffer were followed. Cells were solubilzed in 0.5 M NaOH, and radioactivity was assessed by liquid scintillation spectroscopy. The binding of the cocaine congener, [^{3}H]2β-carbomethoxy-3β-(4-fluorophenyl)tropane ([^{3}H]CFT, also designated [^{3}H]WIN35,428) to DATs on intact cell membranes was determined by incubating cells with the same buffer containing 10 nM [^{3}H]CFT (87 Ci/mmol, Dupont-New England Nuclear, Boston, MA) for 120 min at 4˚C. The parallel incubations using the identical numbers of untransfected CV-1 cells were done to provide estimates of nonspecific uptakes or bindings.

To obtain informations as to the effects of phosphorylation on DAT functions, the uptake (or binding) activities were assessed by pretreating the DAT-expressing cells at 37˚C on the concentration- or time-course manners with each of phorbol 12-myristrate 13-acetate (PMA, a PKC activator) (Castagna *et al.*, 1982; Nishizuka, 1984; Brasseur *et al.*, 1985), staurosphorine (a nonspecific protein kinase inhibitor), calphostin C (a specific PKC inhibitor) (Kobayashi *et al.*, 1989), forskolin (a PKA activator), and EGTA (Ca^{2+}-chelating agent, a inhibitor of CaM kinase II). PMA was phurchased from Calbiochem (La Jolla, CA), and staurosphorin, calphostin C and forskolin were purchased from Sigma (St. Louis, MO).

Results

CV-1 cells, transfected with pRc/CMV-DAT, stably expressed a high affinity DAT under normal conditions with no experimental manipulation. Stable transfectant cells showed 30-50-folds higher dopamine uptake than nontransfectants in dopamine uptake assays. Most of the general characteristics of this transporter were similar to those of DATs, reported in other papers. K_m of dopamine transporting (991.6 nM) is nearly identical to that of expressed-rat DAT (885 nM, Kilty *et al.*, 1991), and inhibitory patterns of dopamine transporting by various psychotropic drugs including cocaine analog are similar to expressed-rat or synaptosomal DAT. However, MPP$^+$ uptake activity of expressed-bovine DAT was significantly lesser than that of expressed-rat

Table 1. Effect of drugs related with protein phosphorylation on DAT functions in CV-1 cells expressing pRc/CMV-DAT. Cells were pre-incubated in the presence of each drugs for 10 min at 37˚C and followed by uptake assays for 10 min ([^{3}H]dopamin uptake) or for 1 h ([^{3}H]MPP$^+$ uptake) at 37˚C. Data represent the mean ± S.D. of three experiments.

	[^{3}H]Dopamine uptake		[^{3}H]MPP$^+$ uptake	
	fmol/10^5 cell/10 min	(% of control)	fmol/10^5 cell/h	(% of control)
Control	65.25 ± 4.34	(100)	4.87 ± 0.38	(100)
PMA (1μM)	20.56 ± 0.87	(31.5)	2.79 ± 0.49	(57.2)
Staurosphorin (5 μM)	100.01 ± 4.10	(153.2)	-	
Calphostin C (0.5 mM)	112.29 ± 11.52	(170.1)	6.81 ± 0.54	(139.8)
PMA + Staurosphorin	93.48 ± 11.43	(143.2)	-	
Forskolin (100 μM)	67.62 ± 5.09	(103.6)	4.13 ± 0.18	(84.8)
EGTA (4 mM)	70.49 ± 12.49	(108.0)	4.55 ± 0.23	(93.4)

DAT (Kitayama *et al.*, 1992a, 1993), and no CFT binding activity was seen. Therefore, the effect of phosphorylation on CFT-binding of DAT could not be done.

Table 1 shows that PKC, but not PKA nor CaM kinase II, modulate the uptake of dopamine and MPP^+. Dopamine uptake was reduced by treatment of PMA, and increased when staurosphorine or calphostin C was added. PMA- effects on MPP^+ uptakes displayed a similar pattern. A dose-response relationships for the effects of PMA on the DAT action demonstrate that dopamine uptake was decreased with increasing concentrations of PMA, reaching a plateau at approximately 1 μM of PMA (Figure 1), but increased infinitely with increasing doses of calphostin C (100 μM - 20 mM). However, no changes were seen by forskolin in the range of concentrations studied (10 nM - 100 μM) (data not shown). The time-effects of preincubation with either PMA or calphostin C on dopamine uptake were shown in Figure 2.

Discussion

It is well known that PKC-mediated phosphorylation plays an important role in the regulation of various receptors for neurotransmitters in the CNS. The existence of putative PKC sites on DAT make it possible to presume the involvement of PKC-mediated phosphorylation in the regulation of DAT functions. The current results show that the functions of DAT are modulated by specific activator and inhibitors of PKC, but not by those of PKA or CaM-kinase II, suggesting that the functions of DAT may be modulated by PKC-mediated phosphorylation. PKC-mediated reductions in dopamine uptake would be expected to enhance the synaptic efficacy of release of a fixed amount of dopamine *in vivo*. Although the current studies cannot directly demonstrate such functions in the dopamine nerve terminals in brain, they do document that such effects are possible

Kitayama reported that efficiencies of dopamine, MPP^+ transport and cocaine binding were differentially altered by the site-directed mutations on some putative transmembrane domains of DAT (Kitayama *et al.*, 1992b; Kitayama *et al.*, 1993). Boja and his colleagues presented controversial finding that more than one cocaine binding site exits in a single dopamine transporter (Boja *et al.*, 1992). These two findings suggest the DAT sites recognizing dopamine, neurotoxin MPP^+, and cocaine may not be located in an identical position, and their mechanisms of actions or regulations can be different, even though they are closely related. Therefore, we tried to find any differences of regulations between the adverse functions (MPP^+ uptake and cocaine binding) and normal physiologic function (dopamine uptake) of DAT by protein phosphorylation. If it turn out to be true, the different regulatory mechanism of DAT functions would be used in the developments of curable or preventable methods for cocaine-addict or Parkinsonism, without impairment of normal DAT function. However, the results in this paper indicate that both of dopamine and MPP^+ transports are modulated by PKC-phosphorylation. It was reported that cocaine binding was also modulated by PMA, although the inhibitory patterns of

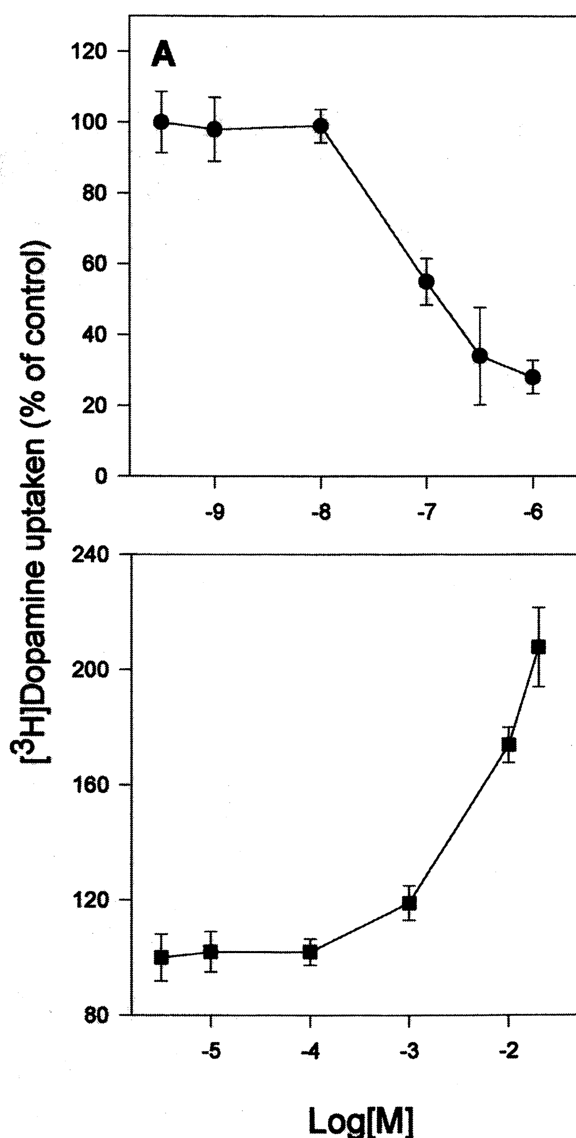

Figure 1. Effects of increasing concentrations of **(A)** PMA or **(B)** calphostin C on dopamine uptake in CV-1 cell expressing pRc/CMV-DAT. Cells were preincubated for 10 min at 37°C with PMA (or calphostin C) at the indicated concentrations. Transport activity was measured in the presence of 100 nM [^{3}H]Dopamine for 10 min at 37°C. Values represent means ± S.D. of three experiments.

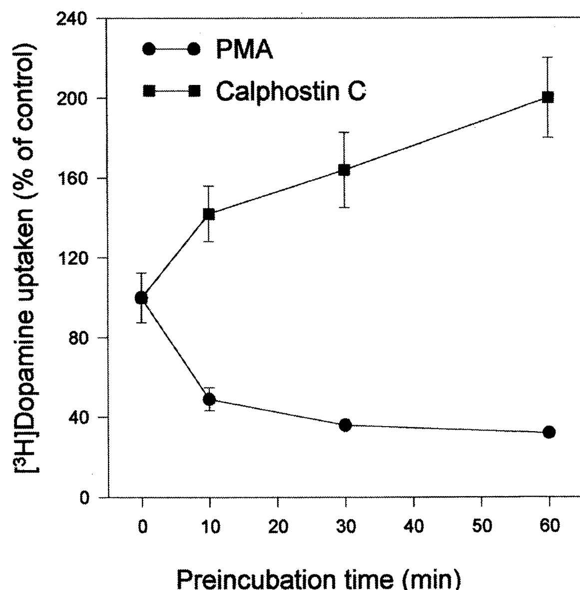

Figure 2. Effects of increasing preincubation-times with PMA or calphostin C on dopamine uptake in CV-1 cell expressing pRc/CMV-DAT. Cells were preincubated with PMA (or calphostin C) for indicated durations at 37˚C, and followed by incubation in the presence of 100 nM [^{3}H]dopamine for 10 min at 37˚C. Values represent means ± S.D. of three experiments.

PMA on dopamine uptake and cocaine binding are slightly different (Kitayama *et al.*, 1994). These findings suggest that all of the DAT functions are modulated by similar PKC-mediated phosphorylation.

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