ELSEVIER

European Journal of Pharmacology 389 (2000) 59–65

ejp

www.elsevier.nl/locate/ejphar

Dopamine transporter antagonists block phorbol ester-induced dopamine release and dopamine transporter phosphorylation in striatal synaptosomes

Rita M. Cowell [b], Lana Kantor [a], G.H. Keikilani Hewlett [a], Kirk A. Frey [c], Margaret E. Gnegy [a,*]

[a] *Department of Pharmacology, University of Michigan School of Medicine, 2240 MSRB III, Ann Arbor, MI 48109-0632, USA*
[b] *Neuroscience Program, University of Michigan School of Medicine, Ann Arbor, MI 48109, USA*
[c] *Department of Nuclear Medicine, University of Michigan School of Medicine, Ann Arbor, MI 48109, USA*

Received 24 September 1999; received in revised form 17 November 1999; accepted 23 November 1999

Abstract

We have reported that inhibition of protein kinase C blocks the Ca^{2+}-independent reverse transport of dopamine mediated by amphetamine. In this study we investigated whether activation of protein kinase C by 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) would mediate dopamine release through the plasmalemmal dopamine transporter. TPA, at 250 nM, increased the release of dopamine from rat striatal slices and synaptosomes while the inactive phorbol ester, 4α-phorbol, was ineffective. The TPA-mediated dopamine release was independent of extracellular calcium and was blocked by a selective protein kinase C inhibitor, Ro31-8220. The dopamine transporter antagonists, cocaine and GBR 12935 blocked the TPA-mediated dopamine release. In addition, cocaine blocked TPA-mediated phosphorylation of the plasmalemmal dopamine transporter. These results suggest that activation of protein kinase C results in reverse transport of dopamine through the plasmalemmal dopamine transporter and the phosphorylated substrate could be the dopamine transporter.

Keywords: Dopamine release; Protein kinase C; Amphetamine; Phorbol ester; Cocaine; Transport, reverse

1. Introduction

Activators of protein kinase C, such as 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), diacylglycerol and arachidonic acid, enhance both depolarization-mediated (Robinson, 1991) and basal release of dopamine (Davis and Patrick, 1990; L'hirondel et al., 1995). Protein kinase C inhibitors, however, have little or no effect on Ca^{2+}- or depolarization-evoked release in the absence of protein kinase C activation (see discussion in Robinson, 1991) suggesting that protein kinase C does not contribute to depolarization-evoked dopamine release in the absence of an accompanying stimulus. It has been concluded that protein kinase C is not required for Ca^{2+}-secretion coupling but could modulate release by phosphorylating a key substrate that regulates depolarization (Robinson, 1991; Coffey et al., 1993).

Release of dopamine without depolarization in response to the protein kinase C activators arachidonic acid (L'hirondel et al., 1995), diacylglycerol (Davis and Patrick, 1990) and TPA (Pozzan et al., 1984) has been demonstrated in striatal synaptosomes and PC12 cells. The diacylglycerol-induced dopamine release in rat striatal synaptosomes was demonstrated to be independent of extracellular calcium (Davis and Patrick, 1990). Further, we and Giambalvo (1992) reported that protein kinase C inhibitors blocked the Ca^{2+}-independent amphetamine-mediated release of dopamine from rat striatal slices. Both 1 μM amphetamine and 250 nM TPA released comparable amounts of dopamine but release in the presence of both agents was non-additive (Kantor and Gnegy, 1998). Since amphetamine requires the plasmalemmal dopamine transporter for its releasing activity (Giros et al., 1996), this

* Corresponding author. Tel.: +1-313-763-5358; fax: +1-313-763-4450.
E-mail address: pgnegy@umich.edu (M.E. Gnegy).

0014-2999/00/$ - see front matter
PII: S0014-2999(99)00828-6

suggests that protein kinase C is required for amphetamine to elicit reverse transport of dopamine through the transporter. It further suggests that direct protein kinase C activation could elicit reverse transport of dopamine through the dopamine transporter.

The objective of this study was to determine whether protein kinase C activation can induce reverse transport of endogenous dopamine through the dopamine transporter. Using TPA as an activator of protein kinase C, we determined that TPA-mediated release from striatal synaptosomes and slices is specifically due to protein kinase C activation and can be blocked by antagonists of the dopamine transporter.

2. Methods

2.1. Preparation of striatal slices

Female Holtzman rats (weight 117–125 g) were killed by decapitation and the striatum was dissected on ice using a brain-cutting block as described (Heffner et al., 1980). The striatal tissue of each rat was sectioned into 1-mm^3 pieces and placed into ice-cold Krebs Ringer Buffer (KRB) containing 125 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, 24.9 mM $NaHCO_3$ and 0.25 M ascorbic acid. The buffer was oxygenated with 95% O_2/5% CO_2 for 1 h.

2.2. Preparation of striatal synaptosomes

A striatal P2 fraction was made from striatal tissue isolated as described above. Tissue was homogenized in 10 volumes of 0.32 M sucrose containing 0.25 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin and 10 μM leupeptin, pH 7.4. Homogenate fractions were centrifuged at $1000 \times g$ for 10 min. The pellet was washed and the combined supernatants were centrifuged at $15\,000 \times g$ for 15 min. The P2 fraction was resuspended in KRB containing 10 μM pargyline to a protein concentration of 1 mg/ml.

2.3. Dopamine release assay

Release of endogenous dopamine was measured in striatal slices and synaptosomes as described previously (Kantor and Gnegy, 1998). Briefly, weighed slices or equal aliquots of synaptosomes were transferred onto Whatman GF/B glass-fiber filters (Maidstone, England) in the appropriate chambers of a Brandel superfusion apparatus (Brandel SF-12; Gaithersburg, MD). Superfusion chambers were maintained at 37°C and medium was perfused through the chambers at a rate of 100 μl/min. Samples were collected at 5-min intervals. After samples were perfused with KRB for 30 min, a 2.5-min bolus of 250 nM TPA was perfused through the sample. In experiments using the protein kinase C inhibitors, either 1 μM Ro31-8220 or 1 μM chelerythrine was included in the perfusate for 20 min before addition of TPA. In experiments using the dopamine transporter antagonists, 10 μM cocaine and 1 μM GBR12935 were perfused simultaneously with the 2.5-min bolus of TPA. The stimulation was terminated by replacing with buffer containing fresh KRB or drug. Collection was continued for an additional 40 min. Results are not corrected for the time that it takes the perfused TPA to reach the slices. The TPA was added at fraction 7; calculating time of delivery, the TPA would reach the sample at fraction 9 and elute at fraction 10. The dopamine content in the perfusate was measured by high-performance liquid chromatography (HPLC) with electrochemical detection using dihydroxybenzylamine as an internal standard (Kantor and Gnegy, 1998). Stock concentrations of Ro31-8220 (1.8 mM), chelerythrine (10 mM), and TPA (10 mM) in dimethylsulfoxide (DMSO) were made and diluted in KRB to final concentrations of 1 μM for the inhibitors and 250 nM for TPA. The final concentration of DMSO in the perfusate was no more than 0.1%.

Statistical analysis was performed on the values attained at the peak TPA response, which is always fraction 10. Statistical significance was determined using one-way analysis of variants (ANOVA) with post-test Tukey–Kramer multiple comparison analysis or by Student's *t*-test.

2.4. Phosphorylation and immunoprecipitation of the dopamine transporter

Striatal synaptosomes (P2 fraction) were preincubated batch-wise with 1 μCi/μl ^{32}P-inorganic phosphate (Amersham,) and 0.5 μM okadaic acid for 45 min at 37°C as described by Vaughan et al. (1997). Aliquots of the synaptosomes (~200 μg) were incubated with 250 nM TPA with or without 10 μM cocaine in a total volume of 100 μl for 45 s at 30°C. The assay was stopped with cold KRB and centrifuged at $4000 \times g$ for 1 min. Pellets were resuspended in RIPAE buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 50 mM sodium pyrophosphate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% sodium deoxycholate, 1 μM pepstatin A, 250 μM phenylmethylsulfonylfluoride, 1 μM leupeptin) and solubilized with rotation for 1 h at 4°C. The mixture was centrifuged at $20\,000 \times g$ for 30 min and the supernatant was precleared with 100 μl of a 20% slurry of Sepharose 4B-immobilized protein G coupled to non-immune immunoglobulin G (IgG). The dopamine transporter was immunoprecipitated following overnight incubation of the precleared material with an antibody specific for the dopamine transporter and coupled to Sepharose 4B-immobilized protein G. The antibody, which was the generous gift of Dr. Allan Levey (Emory University, Atlanta, GA), was produced to the N-terminal fragment of the human dopamine transporter but efficiently recognizes rat dopamine transporter, based on immunoblots and immunocytochemistry

in nigrostriatal-lesioned rats rat dopamine transporter (Hersch et al., 1997; Ciliax et al., 1999).The immunoprecipitated dopamine transporter was pelleted at 4000 × *g* for 1 min and washed 3 times with 50 mM Tris buffer, pH 8.3. Sodium dodecyl sulfate sample buffer was added to the pellets and the immunoprecipitated material was applied to an sodium dodecylsulfate gel containing 8.75% polyacrylamide. One major band at 80 000 Da was obtained from the immunoprecipitation. Autoradiograms were analyzed by densitometry using a Hoeffer GS300 scanning densitometer and Gaussian integration of the peak was determined using the accompanying Hoeffer software.

2.5. Drugs

TPA, 4α-phorbol, Ro31-8220, GBR12935 and chelerythrine were purchased from Calbiochem (La Jolla, CA). Cocaine was the generous gift of Dr. James Woods, Department of Pharmacology, University of Michigan.

3. Results

3.1. TPA-induced dopamine release is independent of extracellular Ca^{2+}

Release of dopamine in response to 250 nM and 1 μM TPA is shown in Fig. 1A. As shown in Fig. 1A, TPA elicits a dose-dependent increase in dopamine release from rat striatal synaptosomes. The release appeared to be independent of extracellular calcium since calcium was not present in the KRB. To further demonstrate the calcium independence of the TPA action, cadmium, a blocker of plasmalemmal calcium channels, was included in the KRB. The data in Fig. 1B, expressed as percent baseline, demonstrate that addition of 10 μM cadmium had no effect on the ability of TPA to induce dopamine release.

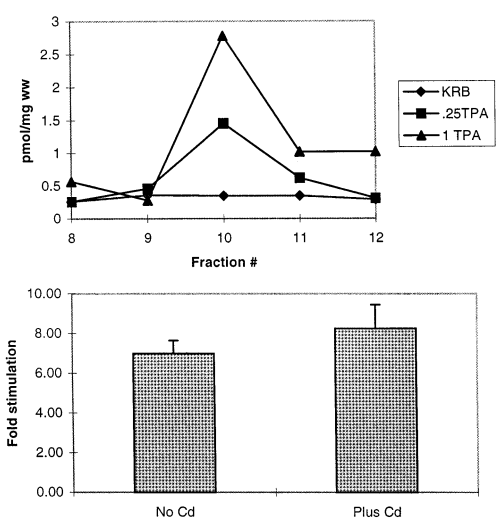

Fig. 1. Ca^{2+}-independent release of dopamine from rat striatal slices by TPA. Rat striatal slices were prepared and endogenous dopamine release measured as described in Section 2. Experiments were conducted in KRB containing no added Ca^{2+}. TPA was added at fraction 7 in a 2.5-min pulse. TPA reached the slices by fraction 9 and dopamine eluted at fraction 10. Results are given as pmole of dopamine per milligram wet weight (pmol/mg ww). (A) Release of dopamine elicited by 0.25 μM and 1 μM TPA. (B) Release of dopamine elicited by 250 nM TPA in the presence and absence of 0.1 mM Cd^{2+} (Cd) expressed as fold stimulation over baseline. $n = 3$. There was no significant difference in TPA stimulation in the absence or presence of Cd^{2+}.

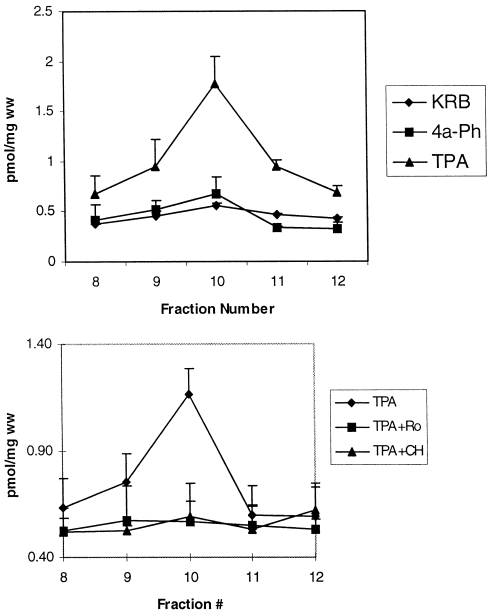

Fig. 2. Role of protein kinase C in TPA-mediated release of dopamine from rat striatal slices. (A) Effect of TPA and the inactive phorbol, 4α-phorbol, on endogenous dopamine release. Both compounds, at 250 nM, were introduced in a 2.5 min bolus at fraction 7. Rat striatal slices were prepared and endogenous dopamine release measured as described in Section 2. $n = 3$. ANOVA on fraction 10, $P = 0.01$. In Tukey's post-hoc test, $p < 0.05$ for 4α-phorbol and KRB as compared to TPA. Results are given as pmole of dopamine per milligram wet weight (pmol/mg ww). (B) Inhibition of TPA-mediated release of endogenous dopamine by the selective protein kinase C inhibitors Ro31-8220 (Ro) and chelerythrine (CH). Ro31-8220 and chelerythrine, at 1 μM, were given 30 min prior to introduction of 250 nM TPA. $n = 3$. ANOVA on fraction 10, $P < 0.03$.

3.2. TPA-induced dopamine release is mediated by protein kinase C

We confirmed that TPA was eliciting dopamine release through activation of protein kinase C by evaluating the effect of TPA and a phorbol (4α-phorbol) which is unable to stimulate protein kinase C activity and by determining the effects of selective protein kinase C inhibitors on TPA-induced dopamine release. The data in Fig. 2A demonstrate that 4α-phorbol was ineffective in eliciting dopamine release from rat striatal slices. To further demonstrate the role of protein kinase C in TPA's action, striatal slices were pretreated with 1 μM Ro31-8220 or 1 μM chelerythrine, two selective inhibitors of protein kinase C. Both inhibitors completely blocked the ability of

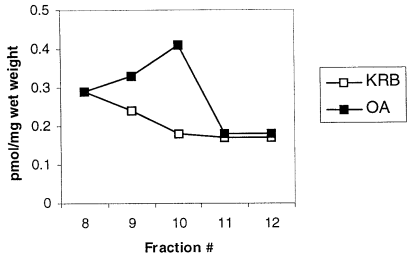

Fig. 3. Okadaic acid (OA) can elicit release of dopamine in the absence of extracellular calcium. Rat striatal slices were prepared and endogenous dopamine release measured as described in Section 2. Okadaic acid, at 100 nM, was given in a 2.5 min bolus at fraction 7.

250 nM TPA to elicit dopamine release from striatal slices (Fig. 2B). If a protein kinase C-mediated phosphorylation is important for reverse transport and release of dopamine, inhibition of phosphatases should elicit reverse transport of dopamine. As shown in Fig. 3, treatment of rat striatal slices with 100 nM okadaic acid elicited a release of dopamine. The experiment was performed in the absence of extracellular Ca^{2+}, which supports a role for release of dopamine through the dopamine transporter.

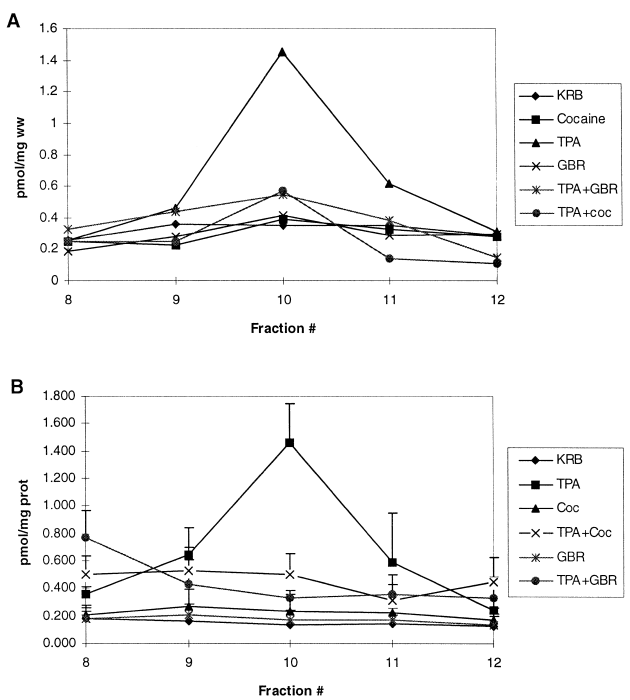

Fig. 4. Inhibition of TPA-mediated release of endogenous dopamine by cocaine and GBR 12935 in rat striatal slices (A) and synaptosomes (B). Rat striatal slices and synaptosomes were prepared and dopamine measured as described in Section 2. Cocaine at 10 μM and 1 μM GBR12935 were given in a 2.5 min pulse at fraction 7 either alone or simultaneously with 250 nM TPA. Results in (A) are given as pmol/milligram wet weight (pmol/mg ww) and in (B) are given in pmol/mg protein (prot). For (A), $n = 1$, For B., $n = 4$. ANOVA on Fraction 10 in synaptosomes, $P < 0.0001$. In Tukey's post-hoc test, $P < 0.001$ for every condition as compared to TPA alone.

3.3. Dopamine transporter antagonists block the ability of TPA to release dopamine from rat striatal slices and synaptosomes

Both cocaine and GBR12935 block amphetamine-mediated release of dopamine through the dopamine transporter (Seiden et al., 1993; Eshleman et al., 1994). If TPA is releasing dopamine through the transporter, its action should be similarly blocked by the dopamine transporter antagonists, cocaine and GBR-12935. To determine this, slices were pulsed with TPA in the presence and absence of 10 μM cocaine or 1 μM GBR 12935. Both drugs were able to inhibit TPA-induced dopamine release from striatal slices (Fig. 4A). The experiment was repeated in a rat striatal synaptosomal P2 preparation to ensure that there were minimal interactions between terminals in the preparation and that an effect of the transporter inhibitors on basal dopamine levels would be minimized. Neither cocaine nor GBR-12935 elicited an increase in baseline values of dopamine and both were able to completely block the dopamine-releasing effect of TPA (Fig. 4B).

3.4. The TPA-increased phosphorylation of the dopamine transporter is blocked by cocaine

Direct phosphorylation of the dopamine transporter by TPA has been previously been demonstrated in striatal

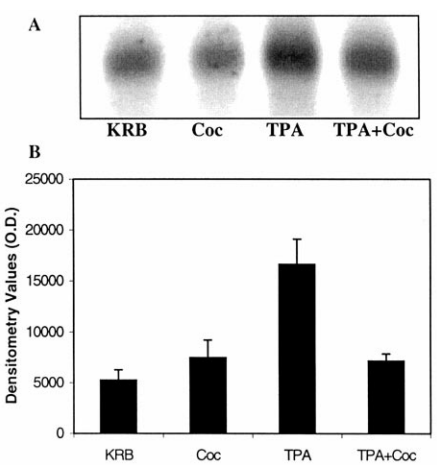

Fig. 5. Cocaine blocks the TPA-mediated phosphorylation of the rat plasmalemmal dopamine transporter. (A) Autoradiogram of the dopamine transporter immunoprecipitated from ^{32}P-labeled striatal synaptosomes. Rat striatal synaptosomes pre-equilibrated with ^{32}P-inorganic phosphate were incubated for 45 s at 30°C with 250 nM TPA, 10 μM cocaine or both as described in Section 2. Dopamine transporter was immunoprecipitated as described in Section 2. The autoradiogram was developed for 5 days. (B) Quantification of autoradiograms by densitometry. Autoradiograms were analyzed by densitometry using a Hoeffer GS300 scanning densitometer and Gaussian integration of the peak was determined using the accompanying Hoeffer software. $N = 3$. * One-way analysis of variance, ANOVA, $P < 0.005$. In post-hoc Tukey analysis, values for KRB, cocaine and TPA + cocaine differed from TPA values at $P < 0.05$. No other values differed from each other.

synaptosomes (Vaughan et al., 1997). We similarly demonstrated phosphorylation of the rat dopamine transporter from striatal synaptosomes in response to TPA (Fig. 5A). One band at 80 000 Da was evident following immunoprecipitation of ^{32}P-labeled striatal synaptosomes with the dopamine transporter antibody and the labeling was increased by incubation with 250 nM TPA. Since cocaine was able to block the ability of TPA to release dopamine from striatal synaptosomes, we examined the effect of cocaine TPA-mediated phosphorylation of the dopamine plasmalemmal transporter. As shown in Fig. 5A, 10 μM cocaine was able to block the phosphorylation of the transporter mediated by TPA. Cocaine alone did not affect transporter phosphorylation. The densitometer values for three separate experiments are shown in Fig. 5B.

4. Discussion

The effect of protein kinase C activation on dopamine transporter activity is a subject of considerable investigation. Protein kinase C activators have been shown to inhibit uptake of dopamine (Kitayama et al., 1994; Copeland et al., 1996; Huff et al., 1997; Zhang et al., 1997). Direct phosphorylation of dopamine transporter by protein kinase C activators has been demonstrated both in dopamine transporter-transfected cultured cells and in striatal synaptosomes. This phosphorylation was correlated with inhibition of dopamine uptake (Huff et al., 1997; Vaughan et al., 1997). Similarly, protein kinase C-mediated phosphorylation of the serotonin plasmalemma transporter, SERT, has been correlated with a reduction of serotonin uptake and reduction of surface SERT (Blakely et al., 1998). Our studies (Kantor and Gnegy, 1998 and this study), however, demonstrate that protein kinase C activation has an additional pronounced effect on the dopamine transporter, that of reversing the transport of dopamine. The fact that the TPA-mediated release of dopamine was independent of extracellular Ca^{2+}, was blocked by dopamine transporter antagonists and was non-additive with amphetamine (Kantor and Gnegy, 1998), strongly suggests that TPA is inducing reverse transport of dopamine.

Although many studies have demonstrated an enhancement of Ca^{2+}-dependent depolarization-mediated release of dopamine by TPA (Robinson, 1991), relatively few have systematically examined the role of protein kinase C in dopamine release in the absence of depolarizing stimulus. Davis and Patrick (Davis and Patrick, 1990) found that diacylglycerol increased basal release of dopamine in striatal synaptosomes in the absence of calcium. Activators of protein kinase C stimulated release of dopamine in PC12 cells at very low intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentrations (Pozzan et al., 1984). Our data, demonstrating TPA's effectiveness in the absence of extracellular calcium and with calcium channels blocked by cadmium clearly demonstrate that TPA is enhancing basal dopamine release independently of vesicular release. The action of TPA is specific for protein kinase C activation since it was blocked by selective inhibitors of protein kinase C and because the inactive compound, 4α-phorbol, failed to elicit release of dopamine. The role for protein phosphorylation in reverse transport is further supported by our data showing that the phosphatase inhibitor, okadaic acid, can elicit the release of dopamine.

The blockade of the dopamine-releasing action of TPA was blocked by the dopamine transporter antagonists strongly suggests that TPA is eliciting release of dopamine through the dopamine transporter. We found that cocaine or GBR 12935 blocked TPA-mediated dopamine release whether they were added at the same time as the TPA (as reported) or added at earlier times (data not shown). Chen and Justice (1998) found that the inhibition profile of cocaine was unrelated to addition time of cocaine. There are several possible mechanisms by which the antagonists could block TPA-induced dopamine release. The simplest possibility is that cocaine and GBR-12935 prohibit the TPA-mediated dopamine efflux from the terminal. GBR-12935 was shown to block spontaneous release of endogenous dopamine through the transporter (Eshleman et al., 1994). Chen and Justice (1998) demonstrated that cocaine binds to the outward conformation of dopamine transporter preventing inward transport of external substrates and thus outward travel of inward substrates. It is possible that binding of cocaine promotes a conformation of transporter such that the protein kinase C substrate site on the transporter is not available. The data showing that cocaine blocked the TPA-mediated phosphorylation of the dopamine transporter support this hypothesis. Ramamoorthy and Blakely (1999) recently reported that cocaine did not alter TPA-mediated phosphorylation of the serotonin plasmalemmal transporters. The substantial differences in their protocol and ours could explain the discrepancy. Their experiments were performed on the serotonin transporter transfected into human embryonic kidney-293 cells. Cocaine was introduced to the kidney cells at 37°C for 20 min prior to a 30 min treatment with TPA. We are investigating phosphorylation of the dopamine transporter in striatal synaptosomes that occurs at very short times and cocaine was added simultaneously with TPA.

The fact that protein kinase C activation can result in both reduction of substrate uptake and enhanced reverse transport is not necessarily conflicting. Protein kinase C could have multiple actions affecting the dopamine transporter and it is entirely conceivable that inward and outward transport could be differentially regulated. For instance, asymmetric inhibition of the glucose and choline transport systems by various compounds has been reported (Krupka and Deves, 1986). Our data that protein kinase C activation elicits reverse transport of the dopamine transporter could be reconciled with the data showing that protein kinase C mediates transporter trafficking in two

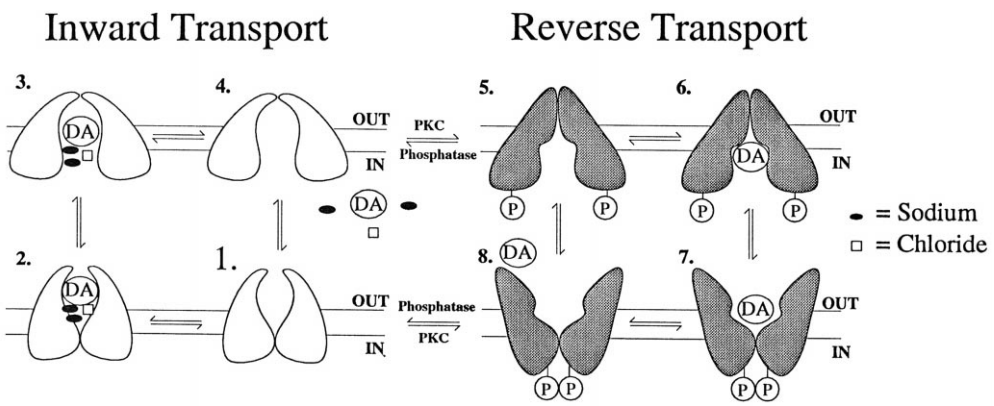

Fig. 6. Schema representing a TPA-induced phosphorylation of dopamine transporter resulting in enhanced outward transport of dopamine. The left panel (1–4) shows the normal activity of the dopamine transporter under normal conditions, whereby extracellular dopamine, sodium ions and chloride ions are transported to the intracellular side of the membrane. In the right panel (5–8), a protein kinase C-mediated phosphorylation of the transporter (or accompanying protein) would change the conformation of the transporter such that its affinity for intracellular dopamine is significantly increased. Inward transport of either dopamine or amphetamine could increase the number of inward facing transporters, expose the relevant protein kinase C consensus sites and permit the necessary conformational change. The presence of dopamine transporter antagonists could stabilize the outward facing transporters and block reverse transport of dopamine.

ways. There could be two separate phosphorylation events mediated by protein kinase C. For instance, low doses of TPA and short incubation times increased proximal tubular bicarbonate and fluid transport in rat kidney while higher TPA doses and incubation times resulted in a decrease in transport (Wang and Chan, 1990). An alternative scenario is that protein kinase C initially mediates reverse transport but the outward conformation of the transporter triggers internalization and trafficking of the transporter(Melikian and Buckley, 1999).

The theory that reversal of transport is due to direct phosphorylation of dopamine transporter is depicted in Fig. 6. Protein kinase C-dependent phosphorylation of the dopamine transporter could affect rate constants for orientation of the transporter in the membrane(Zhang et al., 1997), shifting the equilibrium of transporter conformation to favor outward as opposed to inward transport. Protein kinase C could be changing the stoichiometry of Na^+ and Cl^- coupling so that reverse transport is favored, as opposed to inward transport.A protein kinase C-dependent phosphorylation of dopamine transporter (or an accompanying protein) could affect rate constants for orientation of the transporter in the membrane (Zhang et al., 1997) and/or affinity of the transporter for ions or intracellular dopamine. In support of this theory, the constitutive leak current associated with the dopamine transporter allows the export of cations and is modulated by protein kinase C (Zhu et al., 1997). On the other hand, alterations in activity of ion pumps, such as Na^+, K^+-ATPase activity, could be involved. A protein kinase C-mediated phosphorylation of Na^+, K^+-ATPase leads to a reduction in enzyme activity (Logvinenko et al., 1996), which could increase internal sodium and enhance outward transport through dopamine transporter.

The actions of amphetamine and TPA on the dopamine transporter are quite similar. Both can block uptake of dopamine and elicit a Ca^{2+}-independent release of dopamine. The dopamine release elicited by both agents can be blocked by dopamine transporter antagonists and protein kinase C inhibitors (Kantor and Gnegy, 1998). The action of amphetamine and TPA in eliciting reverse transport of dopamine could reflect similar mechanisms. We have postulated that the action of amphetamine in reversing dopamine transport is dependent upon protein kinase C (Kantor and Gnegy, 1998). Protein kinase C inhibitors blocked amphetamine-mediated dopamine release from rat striatum (Kantor and Gnegy, 1998) and nucleus accumbens (Vezina and Kim, 1999) and attenuated the ability of amphetamine to elicit locomotor activity when injected into rat nucleus accumbens (Vezina and Kim, 1999). Interestingly, we have data suggesting that amphetamine action can elicit phosphorylation at the protein kinase C substrate site of growth associated protein-43 (GAP-43) (Iwata et al., 1996, 1997) suggesting that amphetamine-mediated protein kinase C activation may lead to the phosphorylation of various intracellular proteins.

In summary, our results demonstrate that protein kinase C activation in striatal nerve terminals can lead to a Ca^{2+}-independent release of dopamine through the plasmalemmal dopamine transporter. These data support our hypothesis that reverse transport of dopamine transporter, especially that mediated by amphetamine, requires a protein kinase C-dependent phosphorylation.

Acknowledgements

Support for these studies came from the Biomedical Research Council, University of Michigan. We would like to thank Dr. Allan Levey (Emory University, Atlanta, GA) for his generous gift of antibody to the plasmalemmal dopamine transporter.

References

Blakely, R.D., Ramamoorthy, S., Schroeler, S., Qian, Y., Apparsundaram, S., Galli, A., De Felice, L.J., 1998. Regulated phosphorylation and trafficking of antidepressant-sensitive serotonin transporter proteins. [Review, 65 refs]. Biol. Psychiat 44, 169–178.

Chen, N., Justice, J.B. Jr., 1998. Cocaine acts as an apparent competitive inhibitor at the outward-facing conformation of the human norepinephrine transporter: kinetic analysis of inward and outward transport. J. Neurosci. 18, 10257–10268.

Ciliax, B.J., Drash, G.W., Staley, J.K., Haber, S., Mobley, C.J., Miller, G.W., Mufson, E.J., Mash, D.C., Levey, A.I., 1999. Immunocytochemical localization of the dopamine transporter in human brain. J. Comp. Neurol. 409, 38–56.

Coffey, E.T., Sihra, T.S., Nicholls, D.G., 1993. Protein kinase C and the regulation of glutamate exocytosis from cerebrocortical synaptosomes. J. Biol. Chem. 268, 21060–21065.

Copeland, B.J., Vogelsberg, V., Neff, N.H., Hadjiconstantinou, M., 1996. Protein kinase C activators decrease dopamine uptake into striatal synaptosomes. J. Pharmacol. Exp. Ther. 277, 1527–1532.

Davis, M.E., Patrick, R.L., 1990. Diacylglycerol-induced stimulation of neurotransmitter release from rat brain striatal synaptosomes. J. Neurochem. 54, 662–668.

Eshleman, A.J., Henningsen, R.A., Neve, K.A., Janowsky, A., 1994. Release of dopamine via the human transporter. Mol. Pharmacol. 45, 312–316.

Giambalvo, C.T., 1992. Protein kinase C and dopamine transport: 2. Effects of amphetamine in vitro. Neuropharmacology 31, 1211–1222.

Giros, B., Jaber, M., Jones, S.R., Wightman, R.M., Caron, M.G., 1996. Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature 379, 606–612.

Heffner, T.G., Hartman, J.A., Seiden, L.S., 1980. A rapid method for the regional dissection of the rat brain. Pharmacol. Biochem. Behav. 13, 453–456.

Hersch, S.M., Yi, H., Heilman, C.J., Edwards, R.H., Levey, A.I., 1997. Subcellular localization and molecular topology of the dopamine transporter in the striatum and substantia nigra. J. Comp. Neurol. 388, 211–227.

Huff, R.A., Vaughan, R.A., Kuhar, M.J., Uhl, G.R., 1997. Phorbol esters increase dopamine transporter phosphorylation and decrease transport V_{max}. J. Neurochem. 68, 225–232.

Iwata, S., Hewlett, G.H.K., Ferrell, S.T., Czernik, A.J., Meiri, K.F., Gnegy, M.E., 1996. Increased in vivo phosphorylation of neuromodulin and synapsin I in striatum from rats treated with repeated amphetamine. J. Pharm. Exp. Ther. 278, 1428–1434.

Iwata, S., Hewlett, G.H.K., Gnegy, M.E., 1997. Amphetamine increases the phosphorylation of neuromodulin and synapsin I in rat striatal synaptosomes. Synapse 26, 281–291.

Kantor, L., Gnegy, M.E., 1998. Protein kinase C inhibitors block amphetamine-mediated dopamine release in rat striatal slices. J. Pharmacol. Exp. Ther. 284, 594–598.

Kitayama, S., Dohi, T., Uhl, G.R., 1994. Phorbol esters alter functions of the expressed dopamine transporter. Eur. J. Pharmacol. Mol. Pharmacol. 268, 115–119.

Krupka, R.M., Deves, R., 1986. Looking for probes of gated channels: studies of the inhibition of glucose and choline transport in erythrocytes. Biochem. Cell Biol. 64, 1099–1107.

L'hirondel, M., Chéramy, A., Godeheu, G., Glowinski, J., 1995. Effects of arachidonic acid on dopamine synthesis, spontaneous release, and uptake in striatal synaptosomes from the rat. J. Neurochem. 64, 1406–1409.

Logvinenko, N.S., Dulubova, I., Fedosova, N., Larsson, S.H., Nairn, A.C., Esmann, M., Greengard, P., Aperia, A., 1996. Phosphorylation by protein kinase C of serine-23 of the $\alpha 1$ subunit of rat Na^+, K^+-ATPase affects its conformational equilibrium. Proc. Natl. Acad. Sci. U.S.A. 93, 9132–9137.

Melikian, H.E., Buckley, K.M., 1999. Membrane trafficking regulates the activity of the human dopamine transporter. J. Neurosci. 19, 7699–7710.

Pozzan, T., Gatti, G., Dozio, N., Vicentini, L.M., Meldolesi, J., 1984. Ca^{2+}-dependent and -independent release of neurotransmitters from PC12 cells: a role for protein kinase C activation? J. Cell Biol. 99, 628–638.

Ramamoorthy, S., Blakely, R.D., 1999. Phosphorylation and sequestration of serotonin transporters differentially modulated by psychostimulants. Science 285, 763–766.

Robinson, P.J., 1991. The role of protein kinase C and its neuronal substrates dephosphin, B-50, and MARCKS in neurotransmitter release. Mol. Neurobiol. 5, 87–130.

Seiden, L.S., Sabol, K.E., Ricaurte, G.A., 1993. Amphetamine: effects on catecholamine systems and behavior. Annu. Rev. Pharmacol. Toxicol. 33, 639–677.

Vaughan, R.A., Huff, R.A., Uhl, G.R., Kuhar, M.J., 1997. Protein kinase C-mediated phosphorylation and functional regulation of dopamine transporters in striatal synaptosomes. J. Biol. Chem. 272, 15541–15546.

Vezina, P., Kim, J.H., 1999. Metabotropic glutamate receptors and the generation of locomotor activity: interactions with midbrain dopamine. Neurosci. Biobehav. Rev. 23, 577–589, [Review] [135 refs].

Wang, T., Chan, Y.L., 1990. Time- and dose-dependent effects of protein kinase C on proximal bicarbonate transport. J. Membr. Biol. 117, 131–139.

Zhang, L., Coffey, L.L., Reith, M.E.A., 1997. Regulation of the functional activity of the human dopamine transporter by protein kinase C. Biochem. Pharmacol. 53, 677–688.

Zhu, S.J., Kavanaugh, M.P., Sonders, M.S., Amara, S.G., Zahniser, N.R., 1997. Activation of protein kinase C inhibits uptake, currents and binding associated with the human dopamine transporter expressed in *Xenopus* oocytes. J. Pharmacol. Exp. Ther. 282, 1358–1365.