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The effect of phosphorylation on amphetamine-mediated outward transport

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Abstract

Amphetamine elicits its locomotor-activating and drug-reinforcing effects by releasing the catecholamines dopamine and norepinephrine into the synapse. Amphetamine is a substrate of the plasmalemmal transporters for both dopamine and norepinephrine. As such, it binds to the transporters in conjunction with Na^+ and Cl^-, facilitating a conformational change leading the transporter to face inward. The subsequent binding of intracellular catecholamine results in an outward transport and release of the catecholamine into the synapse. Both inward and outward transport through the catecholamine transporters are regulated by protein kinases, particularly protein kinase C, but the effect of the enzyme on the two processes appears to be asymmetric. The purpose of this review is to discuss the evidence showing that protein kinase C activation facilitates outward transport through the catecholamine plasmalemmal transporters which may mediate amphetamine action in intact tissue.

Keywords: Dopamine transporter; Norepinephrine transporter; Protein kinase C; Dopamine release; PC12 cell; Striatum

1. Introduction

Amphetamine, a drug with a high abuse liability, is a substrate of the plasmalemmal transporters for the catecholamines dopamine and norepinephrine. Central to its behaviorally reinforcing and psychomotor activating effects is its ability to elicit outward transport of dopamine and norepinephrine through their respective plasmalemmal transporters into the synaptic cleft (Koob and Nestler, 1997). It is advantageous, then, to clearly understand the mechanism of action of amphetamine in eliciting the reversal of the catecholamine transporters and the factors that can affect this process.

Both the norepinephrine and dopamine transporters have a high affinity for amphetamine (Wall et al., 1995). As a substrate, amphetamine binds to the catecholamine transporters in the presence of Na^+ and Cl^-, elicits an inward current, and promotes inward transport. The inward current elicits an accumulation of intracellular Na^+ which promotes binding of intracellular dopamine, reversal of the transporter, and the release of dopamine extracellularly (Rutledge, 1978; Sammet and Graefe, 1979; Bonisch, 1986; Sitte et al., 1998; Khoshbouei et al., 2003). Amphetamine also functions to increase synaptic dopamine by competitively blocking dopamine uptake. Both catecholamine transporters function by a coupled carrier-mediated process, such that inward transport should proportionally lead to outward transport. A substance that inhibits inward transport, then, would be expected to subsequently inhibit outward transport. It is possible, however, for a carrier to function asymmetrically and for substances to activate or inhibit the carrier asymmetrically (Stein, 1990).

A seeming asymmetry exists when one examines the effect of phosphorylation, especially protein kinase C-mediated phosphorylation, on inward and outward transport through the dopamine and norepinephrine transporters. As described in more detail elsewhere in this volume, activation of protein kinase C results in a decrease in uptake of catecholamines through the dopamine and norepinephrine transporters largely as a result of internalization of the transporter (for a review of this literature, see Zahniser and Doolen, 2001; Zhang et al., 1997; Reith et al., 1997). On the contrary, activation of protein kinase C can increase outward transport through the dopamine and norepinephrine transporters. Similarly, inhibition of protein kinase C inhibits amphetamine-mediated outward transport through both transporters.

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The purpose of this review is to discuss the effect of protein phosphorylation, primarily protein kinase C-mediated phosphorylation, on amphetamine-mediated dopamine release through the catecholamine transporters.

2. Effect of protein kinase C inhibitors on amphetamine-mediated dopamine release

2.1. Release through dopamine transporters in rat brain

There is an extensive literature on the ability of protein kinase C activation to potentiate exocytotic release of catecholamines (Robinson, 1991; Vaughan et al., 1998). Recently, however, evidence has emerged demonstrating an effect of protein kinase C on external Ca^{2+}-independent transporter-mediated catecholamine release. Davis and Patrick (1990) originally reported that the protein kinase C activators, 1-oleoyl-2-acetylglycerol and *sn*-1,2-dioctanoylglycerol, increased the release of [^{3}H]DA from striatal synaptosomes in the absence of extracellular Ca^{2+}. Since exocytosis requires extracellular Ca^{2+} but dopamine transporter-mediated outward transport does not, it is possible that the protein kinase C-mediated dopamine efflux could have been carrier-mediated. A role for protein kinase C in amphetamine-mediated outward transport was first delineated by the work of Giambalvo (1992a,b). Doses of amphetamine that elicit the release of dopamine, given either in vivo or in vitro in synaptoneurosomes, increased particulate protein kinase C activity in rat striatum and Ca^{2+}- and lipid-dependent phosphorylation of proteins. The in vivo effect was blocked by α-methyl-*p*-tyrosine but was enhanced in the presence of sulpiride. Experiments designed to inactivate the receptors indicated that this effect was not dopamine receptor-mediated. It was stated, but not shown, that a low concentration of phorbol 12-myristate 13-acetate, PMA, which activates protein kinase C, increased the amphetamine-mediated release of dopamine twofold. Moreover, the author found a significant correlation between the IC_{50} for inhibition of protein kinase C activity and the IC_{50} for the inhibition of amphetamine-mediated dopamine release by the protein kinase C inhibitors. These two pieces of information were highly compelling but not presented in any experimental detail.

In an investigation of the effect of repeated amphetamine on phosphorylation of presynaptic proteins, Gnegy et al. (1993) found that acute, in vivo, amphetamine increased the phosphorylation of growth-associated protein-43 (GAP 43, also named neuromodulin, F1, p50). GAP 43 is a neural-specific protein that binds calmodulin, actin, and G_o (Skene, 1990; Coggins and Zwiers, 1991) and is specifically phosphorylated by protein kinase C at serine-41 (Alexander et al., 1988). Use of a site-specific antibody revealed that amphetamine, given either in vivo or in a synaptosomal preparation, increased the immunoreactivity for phosphoser-41-GAP 43 (Iwata et al., 1996, 1997a,b). These data and those of Giambalvo (1992a,b) demonstrate that amphetamine can increase the activity of protein kinase C and phosphorylation of selective protein kinase C substrates in rat striatum.

The activating effect of amphetamine on protein kinase C activity appears to be related to its ability to elicit outward transport of dopamine. Three different protein kinase C inhibitors of different classes, chelerythrine, Ro31-8220, and calphostin C, nearly completely inhibited dopamine release elicited by 1 μM amphetamine in perfused rat striatal slices and synaptosomes (Kantor and Gnegy, 1998; Cowell et al., 2000). Amphetamine, especially at the concentration of 10 μM, has been demonstrated to reduce dopamine uptake into synaptic vesicles and increase cytosolic dopamine, promoting outward transport (Sulzer et al., 1995; Floor and Meng, 1996) and is reported to stimulate exocytotic release from chromaffin cells (Mundorf et al., 1999). The ability of protein kinase C inhibitors to block amphetamine-mediated dopamine release was related to carrier-mediated and not exocytotic dopamine release or synaptic vesicular dopamine for several reasons. Neither the amphetamine-mediated dopamine release nor the inhibition by the protein kinase C inhibitors was dependent upon extracellular Ca^{2+}. Depletion of vesicular dopamine by pretreating the rats with reserpine did not alter the ability of the protein kinase inhibitors to block amphetamine-mediated dopamine release.

PMA, the activator of protein kinase C, increased the outward transport of endogenous dopamine when added to the perfusion medium in the absence of amphetamine. Moreover, the PMA-dependent dopamine release was independent of extracellular Ca^{2+} and was blocked by dopamine transporter blockers cocaine and GBR12935 (Cowell et al., 2000). A schema representing a protein kinase C and/or amphetamine-mediated phosphorylation resulting in enhanced outward transport of dopamine is shown in Fig. 1.

The role of protein kinase C in amphetamine action was validated by examining the ability of protein kinase C inhibitors to alter amphetamine-mediated locomotor behavior in the rat. Intra-accumbal injection of Ro31-8220 prior to an intra-accumbal injection of amphetamine inhibited the amphetamine-mediated locomotor behavior (Browman et al., 1998). Ro31-8220 inhibited amphetamine-mediated dopamine release from nucleus accumbens slices just as in striatal slices.

Although the protein kinase C inhibitors blocked substrate-mediated outward transport, they did not block inward transport (Kantor and Gnegy, 1998). In fact, they slightly increased inward transport of [^{3}H]dopamine. As described above, PMA treatment reduces [^{3}H]dopamine uptake by enhancing the internalization and trafficking of the transporter (see Zahniser and Doolen, 2001). In fact, amphetamine and other transporter substrates, including dopamine, will reduce uptake by increasing internalization of the transporter (Saunders et al., 2000; Gulley et al., 2002).

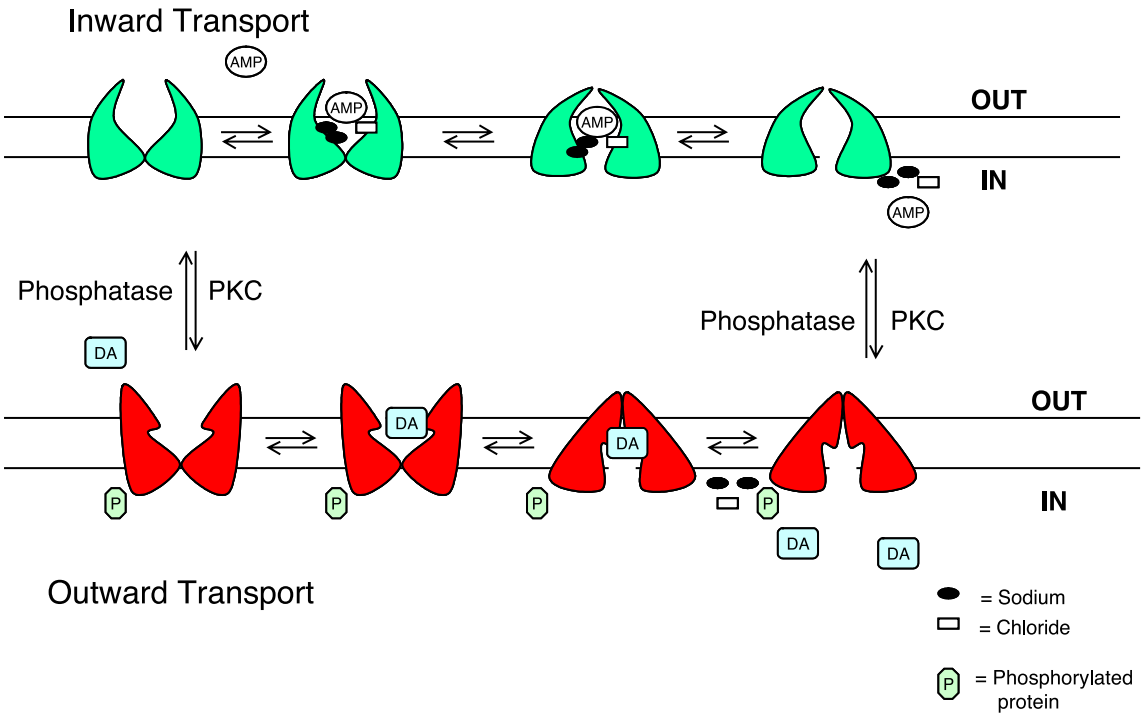

Fig. 1. Schema representing the effect of amphetamine and/or protein kinase C-mediated phosphorylation on outward transport of dopamine through the dopamine transporter. The top panel shows the physiological activity of the dopamine transporter under normal conditions, whereby extracellular substrate [normally dopamine (DA), but in this case, amphetamine (AMP)], sodium ions, and chloride ions are transported to the intracellular side of the membrane. Inward transport of amphetamine would increase the number of inward-facing transporters, expose the relevant protein kinase C substrate sites, and permit the necessary conformational change. In the lower panel, a protein kinase C-mediated phosphorylation of a substrate protein (P, the transporter or an accompanying protein) would change the conformation of the transporter such that more dopamine can bind to the inward-facing transporter. The presence of dopamine transporter antagonists could stabilize the outward-facing transporters and block reverse transport of dopamine.

2.2. Release through norepinephrine transporters in PC12 cells

The affinity of amphetamine at the norepinephrine transporter is as great or even greater than at the dopamine transporter (Giros et al., 1994; Wall et al., 1995; Rothman et al., 2001). The two transporters have a 66% homology (the human transporters) and a very similar pharmacological profile, especially for substrates (Giros and Caron, 1993). Therefore, it is conceivable that the ability of protein kinase C inhibitors to inhibit amphetamine-mediated reverse transport by the norepinephrine transporter would mirror that by the dopamine transporter. This was investigated by Kantor et al. (2001) and was found to be true. Rat pheochromocytoma PC12 cells are an excellent cell system with which to investigate catecholamine-transporter-mediated outward transport. PC12 cells synthesize and store dopamine and norepinephrine and express the norepinephrine transporter (Friedrich and Bonisch, 1986; Apparsundaram et al., 1998b; Cowell et al., 2000). Measurement of release of endogenous dopamine, rather than norepinephrine, is suitable in the PC12 cells because they contain more dopamine than norepinephrine (Greene and Tischler, 1976), both dopamine and amphetamine are excellent substrates for the norepinephrine transporter (Zhu and Hexum, 1992; Gu et al., 1994; Wall et al., 1995), and dopamine is readily released in PC12 cells in response to depolarization (Kittner et al., 1987) and amphetamine (Sulzer et al., 1995; Kantor et al., 2001). Kantor et al. (2001) found that the regulation of amphetamine-mediated outward transport in the PC12 cells by the protein kinase C inhibitors and activators was the same as that for the dopamine transporter in the rat striatum. Pretreatment of the cells with inhibitors of protein kinase C or down-regulation of the protein kinase C by long-term PMA treatment completely inhibited amphetamine-mediated dopamine release in the perfused cells. Moreover, as with the dopamine transporter, PMA increased the release of dopamine from the PC12 cells. Neither the amphetamine-mediated nor the PMA-mediated dopamine release was inhibited by depletion of vesicular catecholamines with reserpine or was dependent upon extracellular Ca^{2+}.

3. Receptor-mediated protein kinase C activation and amphetamine-mediated dopamine release

An interesting series of experiments from Werling's laboratory demonstrated potentiating effects of receptor-mediated protein kinase C activation on amphetamine-mediated dopamine release. Sigma2 receptor agonists have been shown to enhance amphetamine-mediated dopamine release during perfusion of a second amphetamine stimulus

by 50% in rat striatal slices and PC12 cells (Izenwasser et al., 1998; Weatherspoon and Werling, 1999; Derbez et al., 2002). The enhancement was blocked by inhibitors of protein kinase C in rat striatum and by inhibitors of Ca^{2+}/calmodulin protein kinase II (CaM kinase II) in PC12 cells. The σ_2-receptor enhancement required extracellular and intracellular Ca^{2+} and was blocked by inhibitors of L- but not N-type Ca^{2+}-channels. Since the σ_2-receptor antagonists did not affect amphetamine-stimulated outward transport in the absence of a σ_2-receptor agonist, amphetamine is not stimulating the σ_2 receptor. Nicotine was reported to enhance amphetamine-mediated dopamine release during a second amphetamine stimulus in perfused slices from rat frontal cortex, again by about 50% (Drew et al., 2000; Drew and Werling, 2001). The nicotinic effects were selective for the frontal cortex and dependent upon extracellular Ca^{2+} entering through L-type Ca^{2+} channels. The nicotinic effect was blocked by inhibitors of protein kinase C but not CaM kinase II.

The effect of receptors coupled to protein kinase C has also been investigated on inward transport, and, predictably, reductions in substrate uptake have been reported. Muscarinic receptor activation reduced inward norepinephrine transport by 30–40% at a maximal time of 30 min in SH-SY5Y cells (Apparsundaram et al., 1998a,b). A short (5 min) incubation with a metabotropic glutamate receptor agonist reduced dopamine uptake by 20% in rat striatal synaptosomes (Page et al., 2001). Both effects were blocked by inhibitors of protein kinase C. The effect of these treatments on amphetamine-mediated outward transport was not assessed.

4. Amphetamine-mediated activation of protein kinase C

The preponderance of evidence cited above suggests that amphetamine itself would activate protein kinase C activity. There are, in fact, a number of studies that have demonstrated an amphetamine-mediated activation of protein kinase C. Direct activation as assessed by an amphetamine-mediated translocation of the catalytic subunit of protein kinase C to the membrane has been demonstrated in rat striatum at concentrations of amphetamine ≥ 1 μM (Giambalvo, 1992a,b; Iwata et al., 1997b), and we have recently demonstrated this in PC12 cells (Park and Gnegy, preliminary data). Injection of rats with 3 mg/kg amphetamine, which serves as a potent inducer of conditioned taste aversion, increases the membrane-to-cytosol translocation of protein kinase C in the parabrachial nucleus (Krivanek, 1997). Further, the substituted amphetamine, 3, 4-methylenedioxymethamphetamine (MDMA), produced a concentration-dependent increase in membrane-bound protein kinase C, as measured by [^{3}H]phorbol 12, 13 dibutyrate binding sites (Kramer et al., 1998). The translocation was blocked by serotonin reuptake inhibitors but not by serotonin receptor antagonists. As stated above, direct phosphorylation of protein kinase C substrates by amphetamine has been demonstrated in vivo and in synaptosomal preparations (Giambalvo, 1992a,b; Iwata et al., 1996, 1997a,b). At this time, it is not known whether other dopamine transporter substrates, including the endogenous substrate, dopamine, would activate protein kinase C.

It is unclear as to how amphetamine or any transporter substrate would activate protein kinase C. However, amphetamine has been reported to increase intracellular Ca^{2+} spikes in neuronal and chromaffin cells (Vislobokov et al., 1993; Mundorf et al., 1999). Recently, Gnegy et al. (2003) have demonstrated amphetamine-mediated increases in intracellular Ca^{2+} using fura-2 measurements in human embryonic kidney 293 (HEK293) cells transfected with the human dopamine transporter and in PC12 cells (Kantor and Gnegy, preliminary results). Although extracellular Ca^{2+} is not required for amphetamine-mediated dopamine release, evidence suggests that intracellular Ca^{2+} is required. Chelation of intracellular Ca^{2+} with the permeable Ca^{2+} chelator 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetra(acetoxymethyl) ester (BAPTA-AM) severely inhibited amphetamine-mediated dopamine release in PC12 cells and rat striatum (Kantor et al., 2001). Chelation of intracellular Ca^{2+} also inhibited amphetamine-mediated inward and outward currents in HEK293 cells transfected with the human dopamine transporter (Gnegy et al., 2003). This effect was very pronounced at more depolarized voltages, suggesting that Ca^{2+} is required for transporter reversal at some membrane potentials (Gnegy et al., 2003). The increase in intracellular Ca^{2+} could serve to activate select Ca^{2+}-dependent protein kinase C isozymes. Inhibitors of the conventional forms of protein kinase C (α, β, and γ isozymes) reduced amphetamine-mediated currents in *Xenopus* oocytes (Doolen and Zahniser, 2002).

Another possibility is that amphetamine could stimulate the formation of oxidative products that would activate protein kinase C. Neurotoxic doses and regimens of amphetamine and methamphetamine have been shown to enhance oxidative products and oxidative damage (Fleckenstein et al., 1997; Lotharius and O'Malley, 2001; Gluck et al., 2001; Park et al., 2002). Recent evidence suggests, however, that lower doses of amphetamine may also result in formation of oxidative products. Direct infusion of 10 μM amphetamine into rat striatum induced hydroxyl radical formation, an effect that may involve activation of glutamate NMDA receptors (Wan et al., 2000). Wolf et al. (2000) found that while a systemic injection of 5 mg/kg amphetamine produced no measurable increase in hydroxyl radical formation, perfusion of hydroxyl radical trapping agents into the ventral tegmental area of rat brain blocked the ability of the amphetamine to increase glutamate efflux. In an interesting study, Yatin et al. (2002) found that dopamine and amphetamine could increase c-*fos* in HEK 293 cells transfected with the human dopamine transporter (hDAT-HEK293 cells) which do not contain dopamine receptors.

The effect occurred when low concentrations of dopamine (100 nM) and amphetamine (200 nM) were incubated with the cells for 1 h so it did not represent an immediate response to transporter stimulation. Since dopamine was much more potent than amphetamine in eliciting the c-*fos* response, the effect was probably not related to outward transport, but could be related to the maximal velocity of inward transport (Sitte et al., 1998). Nevertheless, the effect was partially blocked by pretreatment with either a protein kinase C inhibitor or an inhibitor of oxidative stress. Activation of protein kinase C by superoxide production has been demonstrated in the hippocampus (Knapp and Klann, 2002).

5. Is the dopamine transporter the substrate for the protein kinase C-mediated phosphorylation?

The dopamine transporter contains several substrate consensus sequences for protein kinase C (Giros and Caron, 1993). Dopamine transporter has proven to be phosphorylated in response to protein kinase C activation endogenously in rat striatal synaptosomes (Vaughan et al., 1997) and when expressed in LLC-PK1 cells (Huff et al., 1997). The phosphorylation can be enhanced by inhibitors of protein phosphatase 1 and 2A (Vaughan et al., 1997). The dopamine transporter appears to be complexed with phosphatase PP2A (Bauman et al., 2000), but the transporter is a direct substrate for protein phosphatase 1 (Foster et al., 2003). The exact site on the dopamine transporter for the protein kinase C-mediated phosphorylation is not known, but evidence suggests that the phosphorylation sites are on serines N-terminal to residue 42 on the cytoplasmic tail of the dopamine transporter (Foster et al., 2002). Although the apparent phosphorylation of the dopamine transporter by protein kinase C correlated with the down-regulation of inward transport and the internalization of the transporter, recent studies cast doubt on the involvement of direct phosphorylation of the dopamine transporter in that function. Mutagenesis of all known protein kinase C-consensus sequences in the dopamine transporter prevented phosphorylation of the transporter but did not abrogate phorbol ester-mediated down-regulation and internalization of the transporter (Chang et al., 2001). Similarly, truncation of the first 22 amino acids of the dopamine transporter abolished protein kinase C-mediated phosphorylation of the transporter but did not alter the ability of the transporter to be down-regulated or internalized (Granas et al., 2003). It is unknown whether the phosphorylation event that alters substrate-mediated outward transport is the same as that eliciting a down-regulation of inward transport, but they could be a continuum of the same activity (see below). On the other hand, the norepinephrine transporter does not contain the N-terminal serines that exist in the dopamine transporter, yet amphetamine-mediated dopamine release is inhibited by protein kinase C inhibitors and PMA increases the Ca^{2+}-independent release of dopamine in PC12 cells containing that transporter.

If the transporter itself is not the target for protein kinase C-mediated phosphorylation, it is possible that a protein associated with the transporter could be the substrate and regulate transporter activity such as syntaxin 1A (Sung et al., 2003) or the protein interacting with C-kinase-1 (PICK1), a protein that can also bind to protein kinase C-α (Torres et al., 2001).

6. Effect of other protein kinases and phosphatase inhibition on amphetamine-mediated outward transport

6.1. CaM kinase II

Although protein kinase C has been demonstrated to have the most pronounced effects on inward and outward transport through the catecholamine transporters, a number of other kinases have been demonstrated to have effects on inward transport. These have been reviewed by Zahniser and Doolen (2001). Effects on substrate-mediated outward transport have been investigated only as regards Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II). Although CaM kinase II has been demonstrated to enhance [^{3}H]dopamine uptake in rat striatum (Uchikawa et al., 1995) and [^{3}H]norepinephrine uptake in PC12 cells (Uchida et al., 1998), no effect of CaM kinase II inhibitors is found on amphetamine-mediated dopamine release from striatum of naive rats (Kantor et al., 1999) or from PC12 cells (Kantor and Gnegy, 2001). Repeated, intermittent treatment of rats or PC12 cells with amphetamine, however, alters the regulation of both the dopamine and the norepinephrine transporter. Repeated, intermittent treatment of rats with amphetamine elicits an enhancement in amphetamine-mediated dopamine release (Robinson and Becker, 1986; Pierce and Kalivas, 1995). The dopamine released in response to a challenge of 1 μM amphetamine is at least doubled in striatal slices from rats pretreated with repeated, intermittent amphetamine as compared to saline controls (Kantor et al., 1999). The enhanced portion of the release depends on extracellular Ca^{2+} and the activity of CaM kinase II, while the nonenhanced portion, equivalent to that from saline-treated or untreated rats, does not (Kantor et al., 1999). Although the enhanced portion of the transporter-mediated dopamine release was dependent on extracellular Ca^{2+}, it was unlikely to be exocytotic in origin since pretreatment of the rats with reserpine did not abolish the enhancement in dopamine release. The same Ca^{2+}- and CaM kinase II-dependent enhancement in amphetamine-mediated dopamine release was demonstrated following repeated treatment of rats with cocaine (Pierce et al., 1998).

Intriguingly, repeated, intermittent treatment of PC12 cells with amphetamine similarly results in an enhancement in amphetamine-induced dopamine release that is dependent

upon extracellular Ca^{2+} and CaM kinase II activity (Kantor and Gnegy, 2001; Kantor et al., 2002). Again, neither reserpine nor tetanus toxin pretreatment abolished the enhancement in amphetamine-mediated dopamine release, suggesting that the extracellular Ca^{2+} dependence does not signal vesicle-mediated exocytosis. No change in inward transport of [^{3}H]dopamine was found in either the rat striatum or PC12 cells following repeated, intermittent amphetamine (Kantor et al., 1999, 2002).

Therefore, repeated treatment with a transporter substrate, amphetamine, elicited a shift in the regulation of the transporter from one that is dependent upon intracellular Ca^{2+} alone to one that is regulated by both intracellular and extracellular Ca^{2+}. Following the repeated amphetamine, however, amphetamine-mediated dopamine release is still completely blocked by inhibitors of protein kinase C (Kantor et al., 1999).

6.2. Effect of phosphatase inhibitors on transporter-dependent dopamine release

If protein kinase activation can promote outward transport, one might expect that inhibition of phosphatase activity would have a similar potentiating effect. This has been demonstrated in bovine retina. Okadaic acid, a specific inhibitor of phosphatases 1 and 2A, potentiated amphetamine- and tyramine-induced dopamine release in the absence of extracellular Ca^{2+} (Bugnon et al., 1995). Okadaic acid alone induced a slight but significant Ca^{2+}-independent release of dopamine in the bovine retina. A Ca^{2+}-independent dopamine-releasing effect of okadaic acid was also demonstrated in rat striatum (Cowell et al., 2000).

7. How can factors such as protein kinase C activation down-regulate inward transport yet increase transporter-mediated outward transport?

7.1. Asymmetric regulation of transport activities

Throughout this review, the reader has been presented with a conundrum: dopamine transporter substrates and PMA will reduce inward transport yet enhance outward transport. The reduction in inward transport appears to be an internalization of the transporter away from the plasma membrane. Although these findings may initially appear to be contradictory, that is not necessarily the case. Although the theory of facilitated exchange diffusion predicts that inward and outward transport would be coupled, in fact, they often appear to be separately regulated. Inward and outward rates of transport at native monoamine transporters can be distinguished by efficacies of different substrates (Sitte et al., 2001), Na^+ dependence (Pifl and Singer, 1999), and Zn^{2+}, which, interestingly, decreases inward transport at DAT but enhances amphetamine-mediated outward transport (Scholze et al., 2002). Sitte et al. (2001) found that there was a poor correlation between the inward transport of substrates and substrate-induced dopamine release; the releasing action of the substrates correlated much better with their ability to induce inward currents. In the case of Zn^{2+}, however, one could postulate that Zn^{2+} promotes an inward facing conformation of the transporter which facilitates binding of dopamine at the intracellular side. Another important consideration is that the dopamine transporter has been demonstrated to be an oligomer (Hastrup et al., 2001; Torres et al., 2003). Although it is not yet clear how oligomerization affects either inward or outward transport,

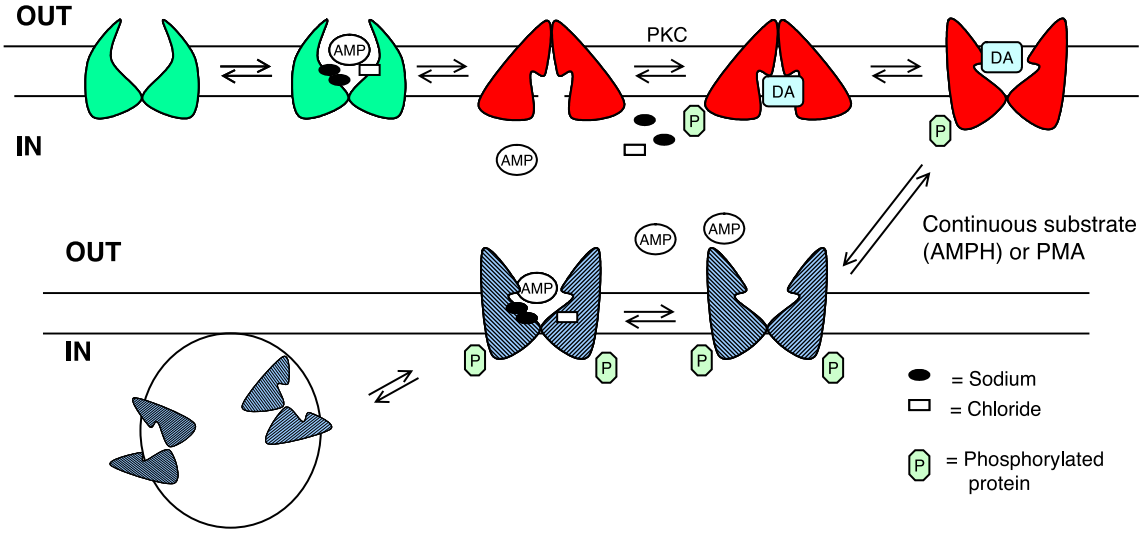

Fig. 2. Schema representing time-dependent effect of amphetamine and/or protein kinase C activation on dopamine transporter activity and regulation. Initial exposure to amphetamine (AMP) or activation of protein kinase C, such as by PMA, would elicit a conformational change in the transporter resulting in an increase in inward-facing transporters. This would lead to enhanced binding of intracellular dopamine (DA) followed by reverse transport of dopamine into the synapse. With time and the continued presence of amphetamine or PMA, the phosphorylation process (P) would lead to a further change in the transporter resulting in its internalization and a decrease in substrate uptake.

Scholze et al. (2002) suggest that oligomerization of the transporter could result in more than one permeation pore that could be separately regulated. Protein kinase C could be a regulatory factor in oligomerization of the transporter.

7.2. Potential time-dependent continuum of activity

The seemingly disparate results concerning the role of protein kinase C activation on inward and outward transport, however, could be also explained by a continuum of activity that begins with the substrate interacting with the transporter. Amphetamine binds to the transporter and initiates the conformational change resulting in inward transport within seconds (Jones et al., 1999; Chen and Justice, 2000). Most experiments examining the effect of substrate or protein kinase C activators on surface transporter and substrate uptake incubate the substrate with the transporter for times from 15 min to 1 h (Saunders et al., 2000; Gulley et al., 2002; Loder and Melikian, 2003). The longer incubation times have the greatest effect on transporter internalization. One can envisage, then, that a short incubation with amphetamine, such as the 2.5-min incubation used by Kantor and Gnegy (1998), would initially drive the transporter to face inward, such that outward transport would be facilitated. This action would be mimicked by protein kinase C activation. Continued presence of substrate or protein kinase C activator would result in an internalization of the transporter, effectively down-regulating its activity. The time- and concentration-differentiated effects of amphetamine on outward transport and internalization of the transporter are depicted in the schema in Fig. 2. On the other hand, the protein kinase C inhibitors would block any activation of the enzyme elicited by amphetamine or reduce an endogenous phosphorylation that could be regulating transporter action. Phosphorylation could thus have a dual effect, whereby a short-term phosphorylation can result in increased outward transport, and a long-term can result in enhanced transporter internalization.

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