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The effect of psychostimulants on [^{3}H]dopamine uptake and release in rat brain synaptic vesicles

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Summary. Amphetamine and its derivatives are psychostimulants active at the plasma membrane monoamine transporters. In the present study we assessed the interaction of parachloroamphetamine, D-amphetamine, fenfluramine and methylendioxymethamphetamine with brain vesicular monoamine transporter using purified rat striatal synaptic vesicles. All four psychostimulants inhibited vesicular [^{3}H]dopamine uptake in a competitive and dose-dependent manner and had no effect on [^{3}H]dihydrotetrabenazine binding. At higher concentrations the drugs enhanced [^{3}H]dopamine vesicular efflux. Parachloroamphetamine was the most potent agent while methylendioxymethamphetamine was the weakest one. The vesicular activities may be relevant to their neurotoxicity.

Keywords: Vesicular Monoamine Transporter (VMAT2), psychostimulants, D-amphetamine, parachloroamphetamine, methylendioxymethamphetamine, fenfluramine, neurotoxicity.

Introduction

Amphetamine and its derivatives are psychostimulats, known by their ability to release monoamines or to block their reuptake from nerve terminals. A number of amphetamine derivatives preferentially release serotonin and are suspected to cause degeneration of serotoninergic nerve terminals. These compounds include methylendioxymethamphetamine (MDMA), parachloroamphetamine (PCA) and fenfluramine. Several mechanisms have been suggested to explain the pharmacologic effects of amphetamine and its derivatives. According to the exchange diffusion model, these substances act principally at the plasma membrane monoamine transporters (Fischer and Cho, 1979; Liang and Rutledge, 1982). They promote a reverse transport of the monoamines and reduce the cytoplasmic concentration of the monoamines. Another mechanism may involve interaction with the brain vesicular monoamine transporter (VMAT2) which is the intraneuronal carrier common to all monoaminergic systems and is responsible for the uptake of monoamines from the cytoplasm into the intracellular storage vesicles. The VMAT mediated transport is coupled to a vacuolar-type H^+ pumping ATPase (Schuldiner, 1994) which creates a pH

gradient across the vesicle membrane. Amphetamine and its derivatives are also known by their ''amphiphilic weak base'' nature (Sulzer and Rayport, 1990). They are sequestered in the intracellular vesicles where they act to reduce the synaptic vesicle pH gradient required for monoamine storage. Amphetamine and its derivatives inhibit the bovine chromaffin granules VMAT at concentrations below those required to dissipate the ATP-generated ΔpH (Rudnick and Wall, 1992; Schuldiner et al., 1993). In the present study we have characterized the interaction of psychostimulants with rat brain VMAT2 using purified synaptic vesicles.

Methods

Materials

[^{3}H]Dopamine (specific activity: 48.2 Ci/mmol) was purchased from NEN Life Science Products, Inc. (Boston, MA). D-amphetamine, MDMA, fenfluramine and para-chloroamphetamine were purchased from Sigma-Aldrich (St. Louis, Mo, USA).

All other chemicals were of highest purity obtainable from regular commercial sources.

Preparation of synaptic vesicles

Synaptic vesicles were prepared from striatum of adult Charles-River rats. Rat brain striatum was homogenized in 10 vol. of ice-cold 0.32 M sucrose containing 10 mM Tris HCl, pH = 7.4, in a glass Teflon homogenizer. The homogenate was centrifuged at 1500×g for 10 min and the supernatant was saved. The pellet was washed once and the combined supernatants were centrifuged at 20000×g for 20 min. The nerve endings in the pellet were disrupted by osmotic shock via homogenization in 10 volumes of 5 mM Tris-HCl buffer (glass-teflon homogenizer) and incubation on ice for 45 min. The homogenate was centrifuged at 20000×g for 20 min and the supernatant containing the released synaptic vesicles was further centrifuged at 62000×g for 40 min.

Vesicular [^{3}H]dopamine uptake

The uptake of [^{3}H]dopamine to striatal synaptic vesicles was performed according to previous reports (Scherman, 1986; Toll and Howard, 1978) with minor modifications. The synaptic vesicles were homogenized in 20 volumes of buffer (100 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM potassium phosphate buffer, pH = 7.4), in a glass Teflon homogenizer. A standard assay contained: 200 µl homogenate, 25 µl buffer or tested compound, 20 µl Na_2ATP (1 mM final concentration) and 25 µl of [^{3}H]dopamine (0.1–5 µM). The tubes were preincubated at 30°C for 10 min, at which time the radioactive monoamine was added. After 1 min incubation, the uptake was terminated by cooling the tubes on ice. The homogenate was filtered under vacuum on GFC filters and radioactivity was counted. Non-specific uptake was measured at 0°C in the presence of 5 µM tetrabenazine. The Ki values for amphetamine and its derivatives were calculated using Linweaver-Burk analysis.

[^{3}H]TBZOH binding

Striatal synaptic vesicles were homogenized in 3 volumes of buffer (20 mM Hepes, 300 mM sucrose), in a glass Teflon homogenizer. A binding assay contained: 50 µl homogenate, 150 µl buffer and 50 µl [^{3}H]TBZOH at final concentration of 3 nM. The tubes were incubated at 25°C for 30 min, at which time the homogenate was added. The homogenate was filtered under vacuum on GFC filters and radioactivity was

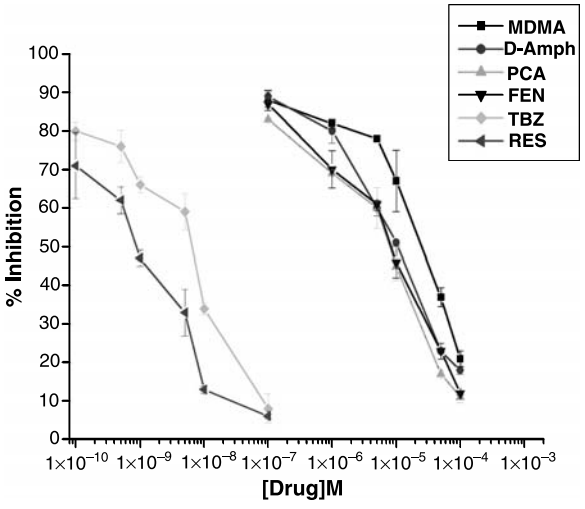

Fig. 1. The inhibitory effect of the drugs on vesicular uptake of [^{3}H]dopamine. Synaptic vesicles were preincubated in the presence of increasing concentrations of the drugs at 30° for 5 min and [^{3}H]dopamine was added for 1 min. Non specific uptake was measured at 0°. Results are expressed as mean ± SEM. *MDMA* Methylenedioxymethamphetamine; *D-Amph* D-amphetamine; *PCA* Parachloroamphetamine; *FEN* Fenfluramine; *TBZ* Tetrabenazine; *RES* Reserpine

counted. Non-specific binding was measured in the presence of 5 μM tetrabenazine.

Retention of previously accumulated [^{3}H]dopamine

Retention of previously accumulated [^{3}H]dopamine was assessed using slight modification of the method described before (Toll and Howard, 1978). Buffer and vesicles were prepared as for vesicular uptake. Two hundred μl sample of synaptic vesicles (about 30 μg protein), 25 μl buffer, 20 μl Na_2ATP (1 mM final concentration) were preincubated for 5 min at 30°C. Twenty-five μl of [^{3}H]dopamine (1 μM final concentration) was added and the incubation was continued for 5 more minutes to allow accumulation of the tritiated dopamine. In order to study the effect of the drugs on the retention of the accumulated [^{3}H]dopamine, increasing concentrations of amphetamine and its derivatives were added, and the incubation was continued for another 5 min. The reaction was stopped by cooling the tubes on ice. The homogenate was filtered under vacuum on GFC filters, and radioactivity was counted.

Table 1. The inhibitory potency (IC_{50}; mean ± SEM) of the various compounds on vesicular [^{3}H]dopamine (1 μM) uptake

Drug	IC50 [M]
Parachloroamphetamine (PCA)	$5.0 \pm 0.6 * 10^{-6}$
Fenfluramine	$7.8 \pm 2.7 * 10^{-6}$
D-amphetamine	$5.2 \pm 0.6 * 10^{-6}$
Methylenedioxymethamphetamine (MDMA)	$1.9 \pm 0.7 * 10^{-5}$
Tetrabenazine (TBZ)	$3.2 \pm 1.9 * 10^{-9}$
Reserpine	$1.8 \pm 1.1 * 10^{-9}$

Three determinations were performed for each compound

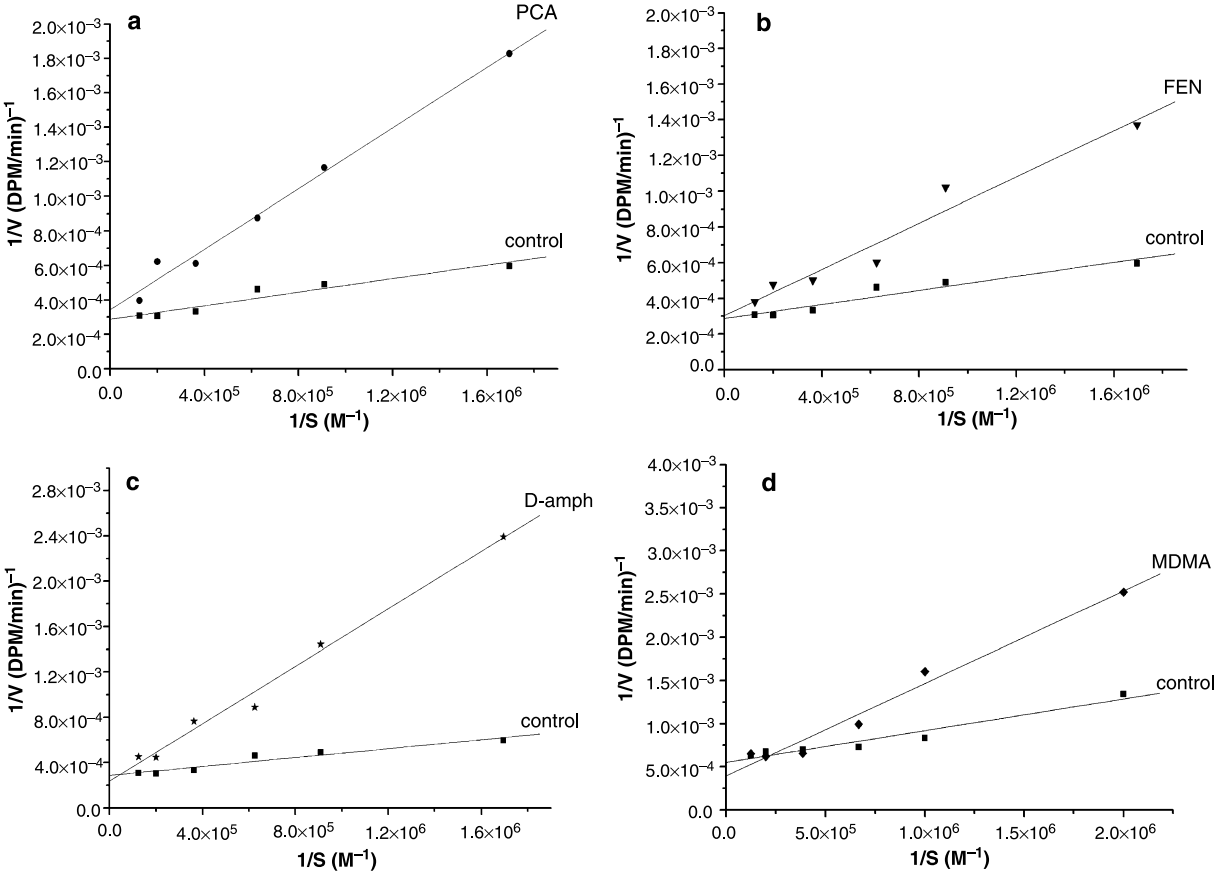

Fig. 2. Lineweaver-Burk analysis of [^{3}H]dopamine uptake into rat striatal synaptic vesicles in the presence and absence of psychostimulants. [^{3}H]Dopamine were in the range of 0.1–5 μM. Uptake was measured as described in Fig. 1. Parachloroamphetamine (PCA), D-amphetamine (D-amph) and fenfluramine (FEN) were at concentrations of 5 μM while methylenedioxymethamphetamine (MDMA) was in concentration of 10 μM

Nonspecific retention was determined by incubation at 0°C.

Results

The effect of psychostimulants on vesicular [^{3}H]dopamine uptake and [^{3}H]TBZOH binding

A dose-dependent inhibition of [^{3}H]dopamine uptake into rat striatal synaptic vesicles by the psychostimulants is depicted in Fig. 1. PCA was the most potent inhibitor, while MDMA was the less active agent (Table 1). All the psychostimulants were relatively very weak inhibitors of the VMAT2 as compared to tetrabenazine and reserpine (Fig. 1, Table 1). All the psychostimulants at concentrations up to 100 μM did not affect [^{3}H]TBZOH binding to the purified synaptic vesicles (data not shown).

Kinetics of vesicular uptake inhibition

Lineweaver-Burk analyses of the uptake kinetics (Fig. 2) indicated that [^{3}H]dopamine vesicular uptake is competitively inhibited by the psychostimulants. The Ki values for the inhibition as calculated from the double reciprocal plots are summarized in Table 2.

[^{3}H]Dopamine retention studies

The effect of the psychostimulants on the retention of [^{3}H]dopamine in the preloaded synaptic vesicles is shown in Fig. 3. The efflux of [^{3}H]dopamine was sensitive to PCA and D-amphetamine but it was much less sensitive to MDMA. Half of the preloaded [^{3}H]dopamine was released in the presence of 10 μM PCA while 100 μM MDMA was required to release 50% of preloaded [^{3}H]dopamine.

Table 2. The inhibitory constants (Ki; mean ± SEM) for vesicular [^{3}H]dopamine uptake of the various psychostimulants

Drug	Ki (M)
Parachloroamphetamine (PCA)	$0.9 \pm 0.1 * 10^{-6}$
D-amphetamine	$1.2 \pm 0.2 * 10^{-6}$
Fenfluramine	$2.2 \pm 0.2 * 10^{-6}$
Methylenedioxymethamphetamine (MDMA)	$1.0 \pm 0.1 * 10^{-5}$

Ki values were calculated from Lineweaver-Burk analyses. [^{3}H]dopamine concentration in the range of (0.1–5 μM)

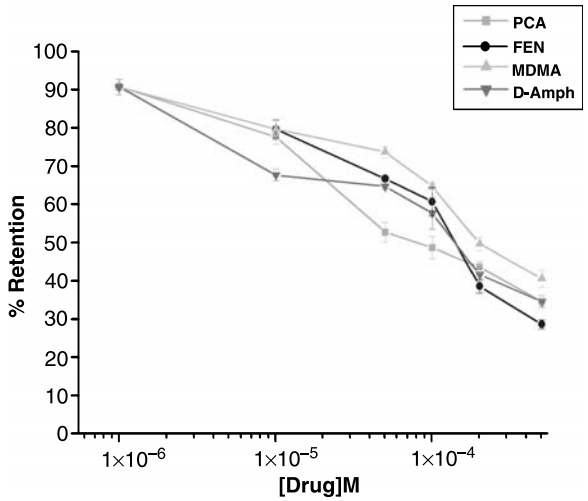

Fig. 3. The effect of the psychostimulants on the retention of [^{3}H]dopamine in preloaded synaptic vesicles. Synaptic vesicles were preincubated at 30°C for 5 min and [^{3}H]dopamine was added for 5 more min. Increasing concentrations of the drugs were added for additional 5 min and the efflux was stoped by cooling the tubes on ice. Non-specific retention was determined by incubation at 0°C. Results are expressed as mean ± SEM

Discussion

In our present study we characterized the interaction between amphetamine and its derivatives with VMAT2 in purified striatal synaptic vesicles. The amphetamine derivatives are known to inhibit the plasma membrane monoamine reuptake as well as their accumulation in synaptic vesicles (Liang and Rutledge, 1982; Sulzer and Rayport, 1990). Our data show that psychostimulants inhibit vesicular [^{3}H]dopamine uptake in a dose-dependent competitive manner similar

to reserpine (Rudnick et al., 1990; Weaver and Deupree, 1982). In contrast, the psychostimulants did not inhibit [^{3}H]TBZOH binding to purified synaptic vesicles. The lack of such inhibitory effect indicates that the tetrabenazine binding conformation is not involved in the mechanism of [^{3}H]dopamine uptake inhibition by the psychostimulants. These results accord a previous observation in bovine chromafin granules (Schuldiner et al., 1995). It is of note, that the VMAT2 inhibitory activity of the psychostimulants is much lower than reserpine and tetrabenazine. The VMAT2 uptake inhibition was achieved at the micromolar range of psychostimulants (Figs. 1, 2). These concentrations are not sufficient to dissipate the pH gradient and to cause vesicular efflux as assessed by the retention studies (Fig. 3) which reflects the content of [^{3}H]dopamine left in the synaptic vesicles following in-vitro exposure to the psychostimulants. This dissociation between the two activities may indicate that the VMAT2 inhibition was achieved using concentrations below those required to induce vesicular efflux. The ability of amphetamine and its derivatives to release the biogenic amines has been shown previously in chromaffin granules (Rudnick and Wall, 1992; Schuldiner et al., 1993; Sulzer et al., 1993). They suggested that amphetamine and its derivatives at low concentrations compete with the substrate of the vesicular monoamine transporter (VMAT) and inhibit in a dose-dependent manner the accumulation of the monoamines in the chromaffin granules. This process is combined with a dissipation of the pH gradient, generated by the vacuolar H^+-ATPase when the weak bases are present in high concentrations. Consistent with that observation, we have demonstrated that psychostimulant-induced efflux of preloaded [^{3}H]dopamine from synaptic vesicles occurs at concentrations above those required to inhibit vesicular uptake of the monoamines. In our study PCA seems to be the most potent inhibitor of VMAT2 as well as the strongest enhancer of [^{3}H]dopamine efflux from synaptic vesicles. MDMA seems to be the less active one. The vesicular depletion of monoamines associated with increased cytoplasmic monoamine concentration may be involved in their neurotoxic effect. Cytoplasmic accumulation of monoamines may lead to generation of intracellular reactive oxygen species (ROS) and induction of neuronal death. Our in-vitro data are consistent with in vivo studies that demonstrated higher neurotoxicity of PCA as compared to MDMA (Colado and Green, 1994). Furthermore, treatment with protective agents that prevented MDMA neurotoxicity fails to prevent PCA neurotoxicity (Colado and Green, 1994; Sprague et al., 1996). It is of note that in contrast to amphetamine, methylphenidate is not neurotoxic to dopaminergic neurons (Yuan et al., 1997). Methylphenidate treatment rapidly increased vesicular dopamine uptake, VMAT2 ligand binding and VMAT2 immunoreactivity in striatal synaptic vesicles and prevented methamphetamine-induced dopaminergic deficits in rats (Sandoval et al., 2003). Since methylphenidate is the widely prescribed psychostimulant for the treatment of attention deficit hyperactivity disorder, characterization of its effect on vesicular dopamine release is warranted. In summary we have shown that amphetamine and its derivatives are inhibitors of VMAT2 and enhancers of [^{3}H]dopamine efflux from synaptic vesicles. Since both vesicular activities are leading to monoamine accumulation in the cytoplasm, they may be relevant to their neurotoxicity.

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