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The mechanism of the releasing action of amphetamine. Uptake, superfusion, and electrophysiological studies on transporter-transfected cells*

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Abstract: Amphetamine analogues are able to induce signs of neurotoxicity in the brain. In order to understand this type of neurotoxicity, the interaction of amphetamine with its molecular targets must be elucidated. These molecular targets are plasmalemmal and vesicular monoamine transporters. We investigated the interaction of amphetamine with these transporters in cells transfected with the respective cDNA. Superfusion and whole-cell, patch-clamp experiments were performed, and the toxicity of substrates of the transporters was studied. Amphetamine was taken up by dopamine transporter-expressing cells in a sodium-dependent and cocaine-blockable manner. Furthermore, it elicited inward currents in these cells concentration-dependently. Correlation of uptake, release, and patch-clamp experiments suggest that ion fluxes induced by substrate-gating on transporters may significantly contribute to the releasing action of amphetamine and of other transporter substrates. Dopamine accumulation into serotoninergic terminals depleted of serotonin by 3,4-methylenedioxymethamphetamine was discussed as a mechanism of Ecstasy-toxicity. This is in agreement with a toxic effect of intracellular dopamine which could be demonstrated on our transporter-overexpressing cells. These results, apart from their relevance for the toxicity by amphetamine analogues, may also have bearings on the mechanisms in neurodegenerative diseases affecting monoamine transmitters.

INTRODUCTION

After administration of amphetamine, methamphetamine, and related psychostimulant drugs, animals develop long-lasting decreases in brain dopamine and serotonin axonal markers. The neurotransmitters themselves (i.e., dopamine and serotonin), their rate-limiting synthetic enzymes (tyrosine hydroxylase and tryptophan hydroxylase), and their transporter sites are lost [1–4]. Chronic self-administration of methamphetamine in humans resulted in reduced levels of dopamine and dopamine transporter in post-mortem striatum [5], and a persistent decrease in brain dopamine transporters was also documented in abstinent human methamphetamine and methcathinone users by means of positron emission tomography with a dopamine transporter ligand [6]. In order to understand this type of neurotoxicity, the interaction of amphetamine with its molecular targets must be elucidated. Amphetamine-related psychostimulants increase extracellular concentrations of catecholamines by displacement from nerve terminals and blockade of uptake systems in the plasma membrane [7]. There is agreement about enhanced neurotransmitter activity at dopamine, noradrenaline, and serotonin receptors, but the mechanism of the facilitated neurotransmission is not yet fully established. Important molecular targets are plasmalemmal and vesicular monoamine transporters. Intraneuronal dopamine has been implicated in neurotoxicity of methamphetamine, which displaces vesicular dopamine into the cytoplasm, resulting in intracellular,

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dopamine metabolism-related, oxidative stress [8]. We examined the actions of psychostimulants in a system rigorously defined by presence or absence of the implicated transport systems. We transfected different cell lines with the human dopamine or noradrenaline transporter and/or the synaptic vesicular transporter cDNA and performed uptake, superfusion, and whole-cell, patch-clamp experiments. Furthermore, we studied the toxicity of substrates of the transporters on transfected cells.

METHODS

Cell culture

COS-7 (African green monkey kidney) cells were grown in Dulbecco's modified Eagle medium with L-glutamine, 4500 mg/L D-glucose, 10% heat-inactivated fetal bovine serum and 50 mg/L gentamicin. Human neuroblastoma (SK-N-MC) and human embryonic kidney (HEK) cells were grown in minimum essential medium with Earle's salts and L-glutamine, 10% heat-inactivated fetal bovine serum and 50 mg/L gentamicin. Cells were grown in 100-mm-diameter tissue culture dishes at 37 °C under an atmosphere of 5% CO_2/95% air.

Cell line transfection

The human dopamine or noradrenaline transporter and the synaptic vesicle amine transporter cDNA were used in the expression vectors pRc/CMV [9] and pCDM8 (SVAT, ref.10), respectively. Cells were transfected by a calcium phosphate procedure [10]. For co-expression 2.5 µg DNA of dopamine or noradrenaline transporter in pRc/CMV plus 7.5 µg SVAT in pCDM8 were transfected into COS-7 cells of which 2×10^6 cells had been seeded into 100-mm-diameter dishes the day before. For stable transfection of SK-N-MC or HEK 293 cells, the calcium phosphate method was modified in the following way: 1×10^6 cells were plated into 100-mm-diameter cell culture dishes two days before transfection. Each 100 mm dish contained 0.75 µg of DNA. The day after transfection, cells were split 1:4 and one day later, selection of cells started using 1 g/L (SK-N-MC cells) or 0.4 g/L G418 (HEK 293 cells) in the medium.

Uptake

For the uptake of [^{3}H]catecholamines and [^{3}H]tyramine (p-hydroxyphenylethylamine) cells were seeded in 24-well plates (5×10^4 cells/well) and, 1 or 2 days later, experiments were performed at 37 °C as described recently [9]. The uptake and binding buffer consisted of (mmol/L): 4 Tris-HCl; 6.25 HEPES; 120 NaCl; 5 KCl; 1.2 $CaCl_2$; 1.2 $MgSO_4$; 5 D-glucose; 0.5 ascorbic acid; pH 7.1. Uptake of amphetamine was generally assessed with cells grown in 6-well tissue culture plates (4×10^5 cells/35 mm well) and amphetamine was determined after precolumn derivatization by HPLC with fluorimetric detection as described recently [11].

Superfusion experiments

Cells were seeded onto poly-D-lysine-coated 5-mm-diameter glass cover slips in 96-well tissue culture plates (2 x 10^4 cells/well). On the following morning cells were loaded with [^{3}H]dopamine, [^{3}H]noradrenaline or [^{3}H]methyl-4-phenylpyridinium (MPP^+) at 37 °C for 45 min (catecholamines) or 20 min (MPP^+) in culture medium. Cover slips were then transferred to small chambers [12] and superfused at 25 °C with 0.7 mL/min of the same buffer as used in uptake experiments. After a washout period of 45 min to establish a stable efflux of radioactivity the experiment was started with the collection of fractions. At the end of the experiment the discs were removed from the superfusion chambers and

immersed in 2 mL of 1% SDS. The radioactivity in the superfusate fractions and the SDS-lysates was determined by liquid scintillation counting. In some experiments, radioactivity in superfusates and cells was analysed for [^{3}H]noradrenaline or [^{3}H]dopamine by combined use of Alumina and Dowex columns, as described by Graefe *et al*. [13]. If not indicated otherwise, release of tritium was expressed as fractional rate, i.e. the radioactivity released during a fraction was expressed as percentage of the total radioactivity present in the cells at the beginning of that fraction.

Patch-clamp experiments

About 100 000 cells were split into poly-D-lysine (0.25 g/L)-coated 35-mm tissue culture dishes the day before. The external (bathing) solution for recordings consisted of (mmol/L): 120 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 20 glucose, 10 HEPES, pH 7.3, 280 mosmol/L. Patch pipettes were either filled with (mmol/L): 140 KCl, 1.59 $CaCl_2$, 10 EGTA, 10 HEPES, pH 7.3, with an osmolality of 300 mosmol/L or with (mmol/L): 130 CsCl, 20 tetra-ethyl-ammonium-chloride (TEA), 0.24 $CaCl_2$, 5 EGTA, 10 glucose, 10 HEPES, pH 7.3, in order to block voltage-activated potassium currents. Recordings were performed in the whole-cell configuration of the patch-clamp technique using an Axopatch 200A patch-clamp amplifier and the pClamp data acquisition system (Axon Instruments Inc.) at ambient temperature (22 ± 2 °C). Cells continuously superfused with bathing solution or solutions containing different concentrations of the substrates dopamine, D-amphetamine, L-amphetamine, tyramine, or with the inhibitor cocaine were routinely voltage-clamped at a holding potential of –70 mV. Alternatively, substances were examined in cells at a holding potential of –40 mV and 150 msec test pulses ranging from –100 to +20 mV. Flow rates in our drug application system are driven by gravity, and switching between different solutions is achieved by solenoid valves to reach plateau concentrations in less than 100 msec. Peak currents were normalized to cell capacitance which ranged from 12 to 80 pF.

Cell viability

Cells were distributed into 12-well plates (SK-N-MC cells, 160 000 cells/well; HEK 293 cells, 80 000 cells/well) and 1–2 d later different concentrations of catecholamines or MPP^+ or vehicle were added to the medium at various times. Cells were recovered by detaching them with trypsin/EDTA, incubated with fluorescein diacetate, and fluorescent cells were counted with a hemocytometer under the fluorescence microscope as described previously [14]. Drugs tested for interference with the dopamine effect were added 1 h (2 h in case of N-acetylcysteine) before dopamine.

RESULTS AND DISCUSSION

COS-7 cells were transfected with the cDNA of the dopamine transporter (DAT-cells) or the vesicular monoamine transporter (VAT-cells) or both of them (DAT/VAT-cells), grown on cover slips, loaded with [^{3}H]dopamine, and superfused in microchambers. In DAT-cells, D-amphetamine induced release, but efflux returned to base-line in spite of the continuing presence of the drug. In DAT/VAT-cells, D-amphetamine had a releasing effect of the same magnitude in terms of radioactivity, but the effect was sustained. In VAT-cells, the absolute effect of D-amphetamine was much smaller. This means the effect of amphetamine is stronger in the presence of a vesicular pool of monoamine; but our results also show that amphetamine can induce dopamine release by solely acting on the plasmalemmal transporter [12].

The effect of D-amphetamine on DAT-cells was concentration-dependent: the minimum effective concentration was 0.1 mM, the maximum was reached at 10 mM, and the effect was reduced to half-maximum at 100 mM. The efflux induced by amphetamine at all concentrations returned to baseline after the switch to amphetamine-free buffer. Addition of D-amphetamine to a buffer superfused over COS-7 cells transfected with the noradrenaline transporter (NAT-cells) and loaded with [^{3}H]noradrenaline

caused a concentration-dependent increase in efflux. The amphetamine effect was bell-shaped in NAT-cells; it started at 10 nM, reached a maximum at 0.1 to 1 µM, and was much lower at 10 mM than at 1 mM and completely absent at 100 mM, but efflux increased to the level attained in the presence of 0.1 or 1 mM when D-amphetamine was removed [15]. Thus, whereas on the dopamine transporter, amphetamine acted as a releasing substance over a wide range of concentrations, on the noradrenaline transporter it behaved as a releasing agent only at high nanomolar to low micromolar concentrations and acted as a pure uptake inhibitor at higher concentrations. It remains to be shown, if this difference of the catecholamine transporters explains the relative resistance of the noradrenergic system against neurotoxic effects of amphetamine-related drugs.

In order to study the interaction of amphetamine with the dopamine transporter more closely we established permanent cell lines expressing the dopamine transporter and examined carrier-mediated release in cells loaded with the metabolically inert substrate MPP^+ [11]. This rules out that release is actually due to effects of releasing substances on metabolism of dopamine. From HEK 293 cells stably expressing the human dopamine transporter, grown on cover slips, loaded with MPP^+, and superfused in micochambers amphetamine can stimulate release in a very reproducible and concentration-dependent manner.

The most popular hypothesis about the mechanism of substrate-induced release is the hypothesis of exchange diffusion [16]: a substrate of the transporter, e.g., amphetamine, is taken up by the transporter and provides transporter sites intracellularly which can then translocate intracellular dopamine in exchange to the exterior of the cell. Following this theory, the higher the uptake of a substrate, the higher its releasing action. In fact, we could demonstrate on our stably transfected cells uptake of amphetamine by the dopamine transporter. Amphetamine was only accumulated by DAT-expressing cells, not by parental HEK 293 cells, the uptake depended on a Na^+ gradient, it was blocked by the inhibitor of the Na^+/K^+-ATPase ouabain or substitution of Na^+ by Li^+ and by cocaine. Tyramine and L-amphetamine also fulfilled the criteria of active uptake by the dopamine transporter. However if the kinetics of initial rates of uptake of the substrates dopamine, tyramine, and D-and L-amphetamine are compared, dopamine had a 4 times higher maximal uptake rate (V_{max}) than tyramine and a 20 times higher than the enantiomers of amphetamine.

By contrast, when we compared the releasing action of these substrates, dopamine, tyramine, and the two enantiomers of amphetamine released from the DAT-cells in a concentration-dependent manner, D-amphetamine was not only the most potent, but also the substance with the highest intrinsic activity, that is the highest maximal effect. These findings do not support a simple mechanism of exchange diffusion considering only substrates of the transporter.

From kinetic studies two Na^+ and one Cl^- are translocated with one dopamine per transport cycle [17]. Since dopamine is a cation at physiological pH, two positive charges should be moved inside per molecule of taken up dopamine, which should result in an inward current calculable from the uptake rates of dopamine. Uptake initial rates of dopamine in our stably transfected cells would predict inward currents in the range of 6 pA. In patch-clamp experiments in the whole-cell configuration at holding potential of –70 mV no currents were observed in untransfected or not-expressing cells, but inward currents of 15 to 70 pA could be elicited by superfusion of DAT-expressing cells by dopamine. Tyramine or the enantiomers of amphetamine also elicited inward-currents and behaved as substrates in this respect. The currents were concentration-dependent, with a rank-order of potency similar to uptake experiments. However, maximal effective concentrations tested on the same cells revealed an about 25% higher maximal effect for D-amphetamine than for the other substrates. Since the current induced by D-amphetamine was higher than that induced by dopamine, it cannot be based on charges co-transported with the translocated substrate. An explanation could be ion-channel properties of the dopamine transporter similar to that reported for other plasmalemmal transporter: substrates gate a kind of channel which lets pass charges uncoupled from substrate-translocation [18].

The same rank-order of intrinsic activity of D-amphetamine, L-amphetamine, dopamine, and tyramine in the induction of release and inward currents suggests a link between release and current

elicited by transporter substrates. A potential link could be intracellular Na^+. Influx of extracellular Na^+ being the trigger for transporter-mediated release was suggested in previous studies [19,20].

Increasing intracellular Na^+ by superfusion of our DAT-expressing HEK 293 cells with the Na^+/K^+-ATPase inhibitor ouabain also resulted in DAT-mediated release since the ouabain-induced efflux was blocked in the presence of cocaine. From the shifts of the current-voltage relation by dopamine or amphetamine on the patch-clamped cells an increase of ionic conductances for either Na^+ or Cl^- can be assumed. Since substrates still induced inward currents when Cl^- was replaced by acetate in the patch pipette, Na^+ most likely carries inward currents. This Na^+ flow might elicit carrier-mediated release. If the substrates dopamine or amphetamine were superfused in the presence of low extracellular Na^+ (lowered to 10 mM) they were not able to enhance efflux of intracellular substrate which was still possible in the presence of low extracellular Cl^-(lowered to 3 mM): under low-Na^+ conditions there might not be enough extracellular Na^+ to fuel substrate-induced Na^+-influx into the superfused cells whereas under conditions of normal extracellular Na^+, but reduced Cl^-, Na^+-influx is high enough for induction of release.

Cells transfected with the DAT cDNA not only allow to demonstrate the decisive role of this plasmalemmal transporter for amphetamine-induced release but also a cytotoxic action of dopamine. A role for dopamine-dependent oxidation in methamphetamine neurotoxicity was implicated in some studies [8,21]. SK-N-MC neuroblastoma cells, stably expressing dopamine uptake at a maximal initial rate of 24 pmol/min/10^5 cells, were exposed to 10 mM dopamine in the medium for 72 h. They suffered a more than 80% cell loss as compared with control treated cells; the cell loss was blocked by the presence of 10 µM mazindol in the medium. The effect of 1 and 10 mM dopamine depended on the expression level of the DAT, that is, it correlated with the V_{max} of uptake. In this low-micromolar range, dopamine had no effect on cells without dopamine uptake. These findings suggest an intracellular site of action.

Inhibition of glycolysis by 2, 5, and 10 mM 2-deoxy-D-glucose in the medium potentiated the cytotoxicity of MPP^+ but not that of the catecholamines. Various antioxidants and monoamine oxydase inhibitors did not interfere with the effect of dopamine. These findings, together with the inactivation-reactivation pattern of aconitase induced by the catecholamines, ruled out mechanisms such as inhibition of the respiratory chain or involvement of oxidative stress. Analysis of cell cycle and DNA fragmentation showed that cell cycle arrest in G_1 and induction of apoptosis were involved in the intracellular effects of catecholamines in the neuronal-type cells.

In conclusion, cell lines heterologeously expressing plasmalemmal or vesicular monoamine transporters make it possible to study the pharmacology of amphetamine-related drug in a very focused way. In this way the molecular sites of action of these types of drugs can be dissected. Furthermore, cells transfected with the plasmalemmal transporter cDNA allows a distinction between an extracellular versus intracellular toxic effect of catecholamines. Neurotoxic mechanisms induced by drugs, but also neurodegenerative processes in various diseases might be modulated by transporter-based compartmentalization of biogenic amines.

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