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Mechanism of the Dopamine-Releasing Actions of Amphetamine and Cocaine: Plasmalemmal Dopamine Transporter versus Vesicular Monoamine Transporter

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SUMMARY

The effects of amphetamine and cocaine were studied in [^{3}H]-dopamine-loaded and superfused COS-7 cells transfected with either the cDNA of the plasmalemmal dopamine transporter ("DAT cells") or the cDNA of the vesicular amine transporter ("VAT cells"), or with both transporters ("DAT/VAT cells"). Amphetamine (0.01–100 μM, added for 4 min of superfusion) led to a concentration-dependent increase in dopamine release in DAT cells, as well as in DAT/VAT cells. The EC_{50} of the effect of amphetamine on DAT cells was 1.1 ± 0.6 μM; the effect on DAT/VAT cells did not reach a plateau in the concentration range tested. With longer exposure to amphetamine, dopamine efflux from DAT cells reached a peak and quickly returned to baseline, in spite of the continued presence of the drug, whereas in DAT/VAT cells and in VAT cells the effect was sustained. Cocaine (up to 100 μM) did not exert any effect of its own in DAT cells or VAT cells but inhibited the amphetamine-induced release of dopamine from DAT cells in a competitive manner. In DAT/VAT cells cocaine and its analogue (−)-2β-carbomethoxy-3β-(4-fluorophenyl)tropane caused an efflux of dopamine resembling that caused by amphetamine but quantitatively much smaller. The rank order of potency was the same as in uptake experiments [(−)-2β-carbomethoxy-3β-(4-fluorophenyl)tropane > cocaine]. The effect of cocaine was mimicked by the reduction of chloride. The results indicate that there is a plasmalemmal component and a vesicular component in the dopamine-releasing action of amphetamine. The releasing action of cocaine is dependent on the existence of a vesicular pool of the neurotransmitter and seems to be linked to inhibition of the plasmalemmal dopamine transporter.

The behavioral effects and the reinforcing properties of the psychostimulant drugs amphetamine and cocaine are suggested to be related to an increase of extracellular dopamine in mesostriatal and mesolimbic areas of the brain (1–3). There is agreement about enhanced neurotransmitter activity at dopamine receptors, but the mechanism of the facilitated dopaminergic neurotransmission has not yet been fully established. Whereas amphetamine and cocaine both interact with the dopamine transport carrier at the plasmalemma of dopaminergic neurons, the involvement of vesicular pools as a site of action is less clear. It has been shown that amphetamine and cocaine block the uptake of dopamine into synaptosomal preparations of dopaminergic brain regions (4–6) and that amphetamine releases dopamine by a mechanism that can be prevented by dopamine uptake blockers (7). However, whereas some studies reported less dopamine release induced by amphetamine from striatal slices in which the vesicular stores had been depleted by reserpine (8, 9), other studies found that the releasing action of only higher concentrations of amphetamine was affected by reserpine (10, 11) or that the action of amphetamine was not at all modulated by reserpine (12). Finally, whereas several authors consider cocaine as an uptake inhibitor devoid of releasing action even at high concentrations (6), other researchers proposed a small but significant effect on release (13), with some authors suggesting that actually all uptake inhibitors are, basically, also releasing drugs (14).

In view of the contradictory data, we made an attempt to examine the corresponding actions of the psychostimulants in a system rigorously defined by the presence or absence of the implicated transport systems. In superfusion experiments on COS-7 cells transfected with the human DAT (15) and/or the SVAT cDNA (16), we hoped to gain insight into the actions of psychostimulants in general and the difference in the actions of amphetamine and cocaine in particular.

Experimental Procedures

Materials. The following drugs were kindly donated or obtained from the sources indicated: 3,4-[7-^{3}H]dopamine (22 Ci/mmol), DuPont

ABBREVIATIONS: DAT, dopamine transporter; CFT, (−)-2β-carbomethoxy-3β-(4-fluorophenyl)tropane; SVAT, synaptic vesicle amine transporter; VAT, vesicle amine transporter; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; CMV, cytomegalovirus.

de Nemours (Dreieich, Germany); cocaine HCl, Dolda AG (Basel, Switzerland); *d*-amphetamine sulfate, SmithKline & French (Welwyn Garden City, Herts, UK); CFT, Research Biochemicals (Natick, MA).

Cell cultures. COS-7 cells were grown in Dulbecco's modified Eagle medium with 580 mg/l L-glutamine, 4500 mg/liter D-glucose, 10% heat-inactivated fetal bovine serum, and 50 μg/ml gentamicin, in 100-mm-diameter tissue culture dishes (polystyrene; Falcon), at 37° under an atmosphere of 5% CO_2/95% air.

Cell line transfection. The human DAT cDNA was used in the expression vector pRc/CMV (17) and the SVAT cDNA was used in the expression vector pCDM8 (16). For transient expression, a modified calcium phosphate transfection was used (18). Cells (2×10^6 cells) were inoculated in 100-mm-diameter dishes 1 day before the transfection procedure. The transfection procedure was started by changing the medium, and 6–7 hr later 10 μg of DNA (2.5 μg of human DAT in pRc/CMV plus 7.5 μg of pRc/CMV, 2.5 μg of human DAT in pRc/CMV plus 7.5 μg of SVAT in pCDM8, or 2.5 μg of pRc/CMV plus 7.5 μg of SVAT in pCDM8) in 450 μl of water were mixed with 50 μl of 2.5 M $CaCl_2$ and 500 μl of a solution containing 0.28 M NaCl, 50 mM HEPES, pH 7.1, and 1.5 mM sodium phosphate and were added dropwise to the 100-mm dish. On the following morning, after removal of the medium, cells were covered with 1 ml of 15% glycerol in PBS, which was immediately diluted with 10 ml of PBS, the whole mixture was removed, and finally the cells were incubated in medium for 7 hr. The cells were then distributed 1:1 either to 48-well plates that contained round coverslips of 5-mm diameter (superfusion experiments) or to 24-well plates (uptake experiments).

Uptake. After 2 days in culture, the cells were used for uptake and superfusion experiments. Uptake for 1 hr was performed by exposing the cells to 0.3 μM [^{3}H]dopamine in culture medium in the absence or presence of 30 μM benztropine or 0.3 μM reserpine, removing the medium after 1 hr, washing the cells twice with 1 ml of PBS/well, solubilizing them in 400 μl of 1% sodium dodecyl sulfate, and estimating the tritium by liquid scintillation counting. Initial rates of uptake were determined in 24-well plates as described previously (17).

Superfusion. Labeling of the cells on coverslips with [^{3}H]dopamine (22 Ci/mmol) was performed in the 48-well plates at 37° for 60 min, in a final volume of 0.5 ml of culture medium (0.3 μM [^{3}H]dopamine; 1 μM [^{3}H]dopamine in the case of sham-transfected cells). The discs were then transferred to small chambers (19) and superfused at 25° at a flow rate of 0.7 ml/min. After a washout period of 50 min the collection of 4-min fractions was started. In a first group of experiments, which were used to obtain concentration-response curves, drugs were added for a 4-min period starting 16 min after commencement of sample collection. In a second group of experiments, the drugs were added after 12 min and their concentration was kept constant until the end of the experiment. The superfusion medium contained 113 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 11 mM D-glucose, 0.5 mM fumaric acid, 5.0 mM sodium pyruvate, 0.57 mM ascorbic acid, and 0.03 mM Na_2EDTA; it was equilibrated with 5% CO_2/95% O_2 and the pH was adjusted to 7.4. In experiments with reduction of chloride, NaCl was replaced by sodium isethionate. At the end of the experiment the radioactivity remaining in the cells was extracted by immersion of the discs in 1.2 ml of 2% (v/v) perchloric acid, followed by sonication. Radioactivity in extracts and collected fractions was determined by liquid scintillation counting (Beckman LS 6500; counting efficiency, 24%).

Calculations. The rate of tritium outflow per minute (fractional rate) was calculated by dividing the amount of radioactivity in a 4-min superfusate fraction by the radioactivity in the cells at the beginning of the corresponding collection period, with subsequent division by 4. Drug-induced overflow of radioactivity was calculated as the difference between total outflow during and after the addition of drug and estimated basal outflow, which was assumed to decline linearly from the sample preceding drug addition to that obtained 4–8 min after drug addition. Drug-induced overflow was expressed as the percentage of the total amount of tritium in the cells at the beginning of drug addition.

Determination of dopamine and 3,4-dihydroxyphenylacetic acid in superfusion fractions. Two 4-min fractions were collected; half of the sample was counted directly by liquid scintillation counting and the other half was extracted with Al_2O_3 and fractionated by HPLC, using an extraction method and a HPLC system described previously (20).

Statistics. All data are given as means ± standard errors; *n* is the number of observations.

Results

Uptake of [^{3}H]Dopamine

COS-7 cells transfected with 2.5 μg of human DAT in pRc/CMV/2×10^6 cells ("DAT cells") and exposed to 0.3 μM [^{3}H]-dopamine in the medium accumulated in 1 hr about 20 times the amount of dopamine accumulated in sham-transfected cells (data not shown). This dopamine transport could be blocked by 30 μM benztropine and was not affected by 0.3 μM reserpine (Fig. 1). COS-7 cells transfected with 2.5 μg of human DAT in pRc/CMV and 7.5 μg of SVAT in pCDM8/2×10^6 cells ("DAT/VAT cells") accumulated in 1 hr 2 times the amount of dopamine accumulated by DAT cells. In the presence of reserpine the level of accumulation in DAT/VAT cells was the same as that in DAT cells (Fig. 1). COS-7 cells transfected with 7.5 μg of SVAT in pCDM8/2×10^6 cells ("VAT cells") did not accumulate significantly more dopamine than sham-transfected cells (VAT cells, 1.30 ± 0.14 pmol/hr/well; sham-transfected cells, 1.24 ± 0.04 pmol/hr/well; mean ± standard error, $n = 4$).

Amphetamine and cocaine inhibited the initial rate of [^{3}H]dopamine uptake (5-min accumulation) with K_i values of 0.29 ± 0.04 and 0.28 ± 0.01 μM, respectively, in DAT cells and 0.70 ± 0.06 and 0.18 ± 0.03 μM, respectively, in DAT/VAT cells (n = 3).

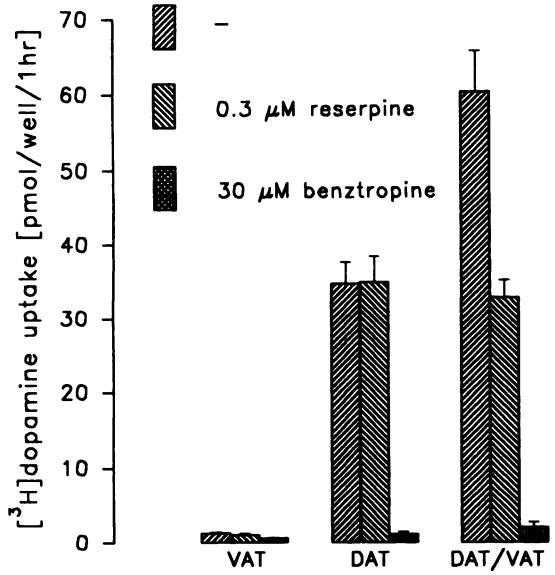

Fig. 1. [^{3}H]Dopamine uptake by VAT cells, DAT cells, and DAT/VAT cells. COS-7 cells transfected with the cDNA for the SVAT (VAT cells), the plasmalemmal DAT (DAT cells), or both transporters (DAT/VAT cells) and distributed in 24-well plates as described in Experimental Procedures were exposed to 0.3 μM [^{3}H]dopamine in the medium for 1 hr in the absence or presence of 30 μM benztropine and 0.3 μM reserpine, washed, and solubilized, and the tritium activity was determined by liquid scintillation counting. Shown is the mean ± standard error of four experiments.

Superfusion Experiments

Spontaneous efflux. The fractional rate of spontaneous efflux of radioactivity during minutes 8–12 after the start of fraction collection amounted to 0.0129 ± 0.0013 min^{-1} ($n = 39$) in DAT cells and 0.00165 ± 0.000075 min^{-1} ($n = 46$) in DAT/VAT cells. These values corresponded to 184 ± 14 and 179 ± 6 cpm/min, respectively. Thus, the amounts of radioactivity released under base-line conditions did not differ significantly, whereas the rate of efflux was about 8 times higher in DAT cells, compared with DAT/VAT cells.

Effects of amphetamine and cocaine. When amphetamine was added for 4 min (one superfusion fraction) to the superfusion buffer, a rapid and reversible increase in efflux of tritium was observed in DAT cells and DAT/VAT cells (Fig. 2A, *inset*). The effect of amphetamine was concentration dependent and reached a maximum at 10 μM, whereas in DAT/VAT cells saturation was not observed at concentrations up to 100 μM (Fig. 2A). The EC_{50} of amphetamine in DAT cells was 1.1 ± 0.6 μM.

Cocaine, when added to the superfusion medium in the same manner as amphetamine, did not change the efflux of tritium in DAT cells (at up to 100 μM) but induced a concentration-dependent dopamine efflux in DAT/VAT cells, which also did not reach saturation (Fig. 2B). The magnitude of the effect in DAT/VAT cells, however, was considerably smaller than the effect of amphetamine in these cells. CFT, a cocaine analogue that was about 10-fold more potent than cocaine in blocking dopamine uptake initial rates (data not shown), induced dopamine efflux from DAT/VAT cells with a significantly higher potency than cocaine. CFT (100 μM) was without effect in DAT cells (data not shown). When the action of amphetamine was tested in DAT cells in the presence of 1 μM or 10 μM cocaine, a shift to the right of the amphetamine concentration-response curve was observed (Fig. 3).

Exposure to amphetamine (1 μM) for 36 min (nine superfusate fractions) revealed a striking difference in tritium efflux between DAT cells and DAT/VAT cells (Fig. 4, A and B). The efflux from DAT cells reached a maximum within 8 min and subsequently returned to base-line in spite of the continuing presence of amphetamine. In contrast, in DAT/VAT cells efflux remained elevated above base-line values after it had reached its peak. In VAT cells exposed to 1 μM [^{3}H]dopamine for 1 hr, amphetamine induced an increase of efflux that, although

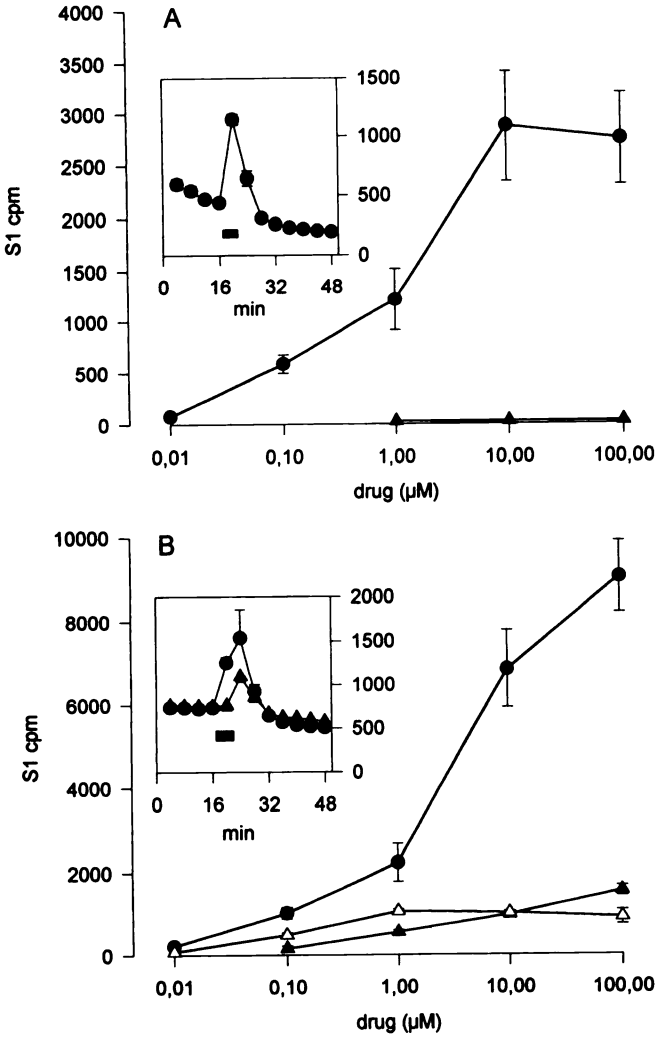

Fig. 2. Concentration-response curves for the effects of amphetamine and cocaine on [^{3}H]dopamine efflux from COS-7 cells. The cells were transfected with the cDNA of the plasmalemmal DAT either alone (DAT cells) (A) or together with that of the SVAT (DAT/VAT cells) (B), grown on 5-mm coverslips, loaded with [^{3}H]dopamine, and superfused. After a 50-min washout period, 4-min fractions were collected. Amphetamine (●), cocaine (▲), or CFT (△) was present from minute 16 to minute 20 of the sampling period. The effects of the drugs are given as cpm released above estimated basal efflux (*S1*) (see Experimental Procedures). *Insets*, time course of the efflux of tritium elicited by 1 μM levels of the drugs. *Symbols*, means ± standard errors of three or four experiments.

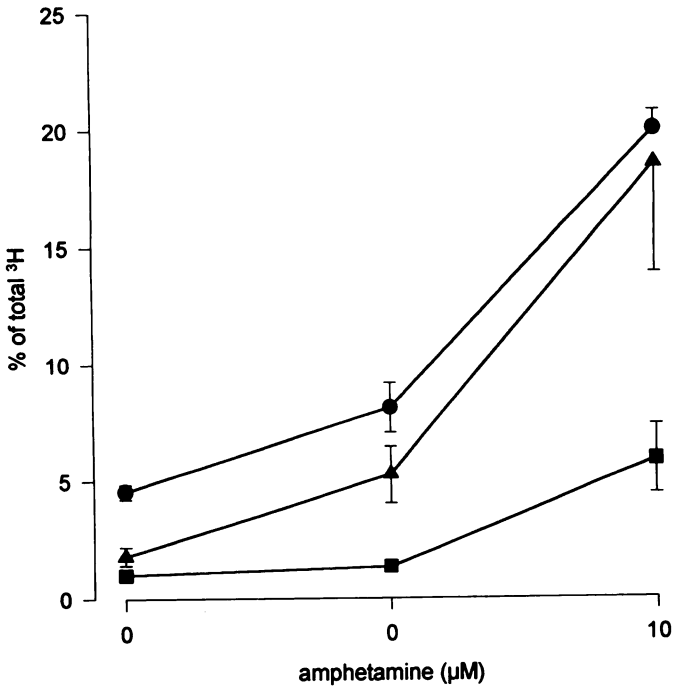

Fig. 3. Effect of cocaine on the amphetamine-induced [^{3}H]dopamine efflux from COS-7 cells. The cells were transfected with the cDNA of the plasmalemmal DAT (DAT cells). Experimental procedures were the same as in Fig. 2. Various concentrations of amphetamine were added during minutes 16–20 of the sampling time, in the absence (●) or presence of 1 μM (▲) or 10 μM (■) cocaine. The effects of amphetamine were calculated as radioactivity released above the estimated base-line and are expressed as percentage of the total radioactivity present in the cells at the beginning of amphetamine stimulation. *Symbols*, means ± standard errors of three or four experiments.

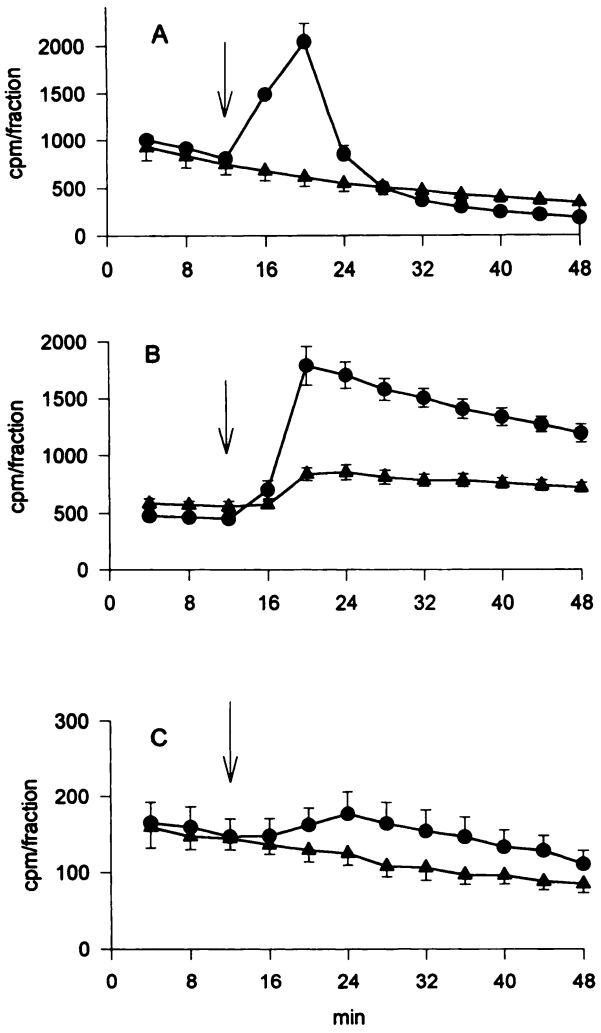

Fig. 4. Effects of amphetamine and cocaine on [^{3}H]dopamine efflux from COS-7 cells. The cells were transfected either with the cDNA of the plasmalemmal DAT alone (DAT cells) (A), with the cDNAs of the plasmalemmal DAT and of the SVAT (DAT/VAT cells) (B), or with the cDNA of the SVAT alone (VAT cells) (C). All cells were grown on 5-mm coverslips, loaded with [^{3}H]dopamine, and superfused. After a 50-min washout period, 4-min fractions were collected, and amphetamine (1 μM in A and B; 10 μM in C) (●) or cocaine (1 μM in A and B; 10 μM in C) (▲) was added to the superfusion buffer at 12 min (*arrows*) and maintained for the rest of the experiment. [^{3}H]Dopamine efflux is shown as cpm/fraction. *Symbols*, means ± standard errors of three or four experiments.

quantitatively smaller than that in DAT/VAT cells, was also step-like (Fig. 4C). Cocaine did not alter the efflux of radioactivity from DAT cells, even when added for a longer period of time, but caused a persistently elevated efflux from DAT/VAT cells. Similarly to the shorter experiment, the effect of cocaine was markedly smaller than the effect of amphetamine. Cocaine was without effect in VAT cells (Fig. 4C).

COS-7 cells that were sham transfected, using the plasmid without transporter cDNA insert, and exposed to 1 μM [^{3}H]-dopamine for 1 hr displayed a modest efflux of tritium that was, however, not affected by the addition of amphetamine or cocaine (data not shown).

The efflux-stimulating action of cocaine in DAT/VAT cells was mimicked by reduction of chloride. Reduction of chloride had no effect on DAT cells (Fig. 5).

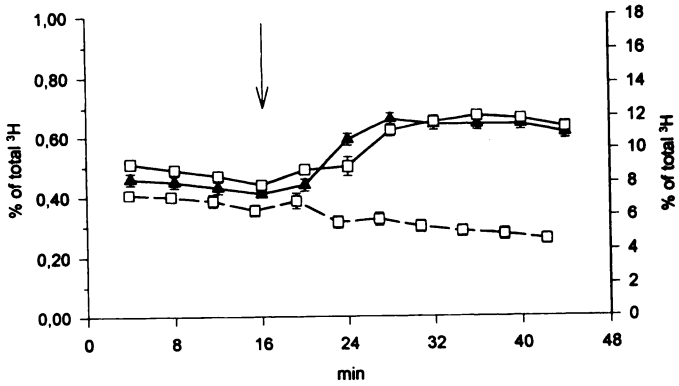

Fig. 5. Effects of cocaine and removal of Cl^- ions on [^{3}H]dopamine efflux from COS-7 cells. The cells were transfected with either the cDNA of the plasmalemmal DAT (*dashed line*, *right ordinate*) or that of both the plasmalemmal DAT and the SVAT (*solid lines*, *left ordinate*), grown on 5-mm coverslips, loaded with [^{3}H]dopamine, and superfused. After a 50-min washout period, 4-min fractions were collected. From 16 min onwards (*arrow*), superfusion was continued in the presence of cocaine (10 μM) (▲) or with reduced chloride (□). [^{3}H]Dopamine efflux is expressed as percentage of the total radioactivity present in the cells at the beginning of each collection period. *Symbols*, means ± standard errors of three or four experiments.

Fractionation of Tritium Efflux by HPLC

The analysis of the superfusates by extraction and HPLC revealed that in DAT cells about 70–80% and in DAT/VAT cells 80–90% of the tritium was present as [^{3}H]dopamine. We therefore refer to the tritium efflux as released dopamine in the Discussion.

Discussion

The results of this study demonstrate that the plamalemmal DAT is involved in the dopamine-releasing action of the prototypic psychostimulant drugs amphetamine and cocaine and the presence of vesicular monoamine transport modifies this effect. COS-7 cells transfected with the cDNA of the plasmalemmal DAT alone or co-transfected, in addition, with the SVAT accumulated dopamine into two different pools. Using our transfection protocols, the two types of cells appeared to have cytoplasmic pools of similar size. Thus, in cells transfected with both transporters reserpine treatment limited the amount of accumulated dopamine to exactly the same level as in cells transfected with the cDNA of the plamalemmal DAT alone. Reserpine had clearly no effect on the plasmalemmal DAT (Fig. 1).

There are two reasons to assume that in our co-transfection experiments cells expressing the DAT most likely also expressed the SVAT. First, with calcium phosphate transfection, cells either take up DNA or stay free of it but they do not differ in their preference for a special type of DNA if the DNAs are of similar size. Second, in our transfection experiments we used an amount of plasmid containing the cDNA for the SVAT that was 3-fold higher than the amount of plasmid containing the cDNA for the plasmalemmal DAT; this procedure increases the probability that cells expressing plasmalemmal uptake also express vesicular uptake (21).

Our data indicate that the SVAT is expressed in COS-7 cells on an intracellular compartment and not on the plasma membrane. Thus, the cells accumulated minute amounts of dopamine if the plasmalemmal DAT was absent or blocked by benztropine (compare VAT cells and DAT/VAT cells in Fig.

1). This means that the SVAT required intracellular dopamine to accumulate it in a reserpine-sensitive compartment.

The spontaneous release of accumulated radioactivity differed between cells expressing the plasmalemmal DAT (DAT cells) and cells expressing both the plasmalemmal DAT and the SVAT (DAT/VAT cells). Whereas DAT/VAT cells began with a total amount of dopamine that was about 2-fold higher than that in DAT cells, they ended with remaining activity that was about 10-fold higher than that in DAT cells. The reason for this difference can be seen in the greater spontaneous release of dopamine in DAT cells, as indicated by a greater drift of the base-line. Obviously DAT/VAT cells have a higher capacity for retaining accumulated dopamine by means of their vesicles, whereas the cytoplasmic pool of dopamine, the only one present in DAT cells, is more easily washed out by superfusion.

The psychostimulant-induced release of dopamine differed between DAT cells and DAT/VAT cells both qualitatively and quantitatively. The qualitative differences were (i) the lack of effect of cocaine on DAT cells but the clear releasing action of cocaine in DAT/VAT cells and (ii) the peak-shaped effect of amphetamine on DAT cells versus the step-shaped release in DAT/VAT cells. In quantitative terms, (i) amphetamine released larger absolute amounts of dopamine in DAT/VAT cells than in DAT cells but (ii) it had a higher potency in DAT cells than in DAT/VAT cells. These differences suggest the following two distinct mechanisms of amphetamine-induced release: (i) reversal of the transport action of the plasmalemmal DAT (6, 7, 10) with a potency similar to its potency in inhibiting dopamine uptake initital rates (in agreement with this hypothesis, the release of amphetamine in DAT cells was inhibited by the transporter blocker cocaine) (Fig. 3) and (ii) release of dopamine from the intracellular vesicular pool (see the dopamine-releasing action from VAT cells), possibly by dissipation of the transmembrane ΔpH that drives biogenic amine uptake into synaptic vesicles (22) or by direct interaction with the substrate binding site of the SVAT (23). The latter effects are assumed to occur at higher concentrations of amphetamine (22, 23), which would agree with the second phase of release starting, as shown in our study, at about 1 μM in DAT/VAT cells (Fig. 2B); the first, "plasmalemmal," part of the amphetamine concentration-response curve would then correspond to the curve observed in DAT cells (Fig. 2A). The alkalinizing action on the vesicular compartment may also be the basis of the dopamine-releasing effect of cocaine in DAT/VAT cells, with a potency much lower than its potency in inhibiting dopamine uptake initial rates. On the other hand, CFT induced dopamine release with a higher potency than did cocaine, reminiscent of its 10-fold higher potency in inhibiting dopamine uptake. This rank order of potency and the fact that reduction of chloride, an ion required for plasmalemmal transport (24), is equieffective with cocaine suggest that blockade of the plasmalemmal uptake of dopamine secreted by recycled vesicles may result in increased efflux of dopamine in superfusion experiments. In agreement with this hypothesis, cocaine could not elicit dopamine release in DAT cells (no vesicles with vesicular amine transport) or in VAT cells (no plasmalemmal transport). The low potency of CFT and cocaine, compared with their potency in uptake experiments, may be due to the nonequilibrium conditions between a small amount of substrate supplied by secreting vesicles and very efficient uptake by the highly expressed DAT.

The effects of amphetamine and cocaine in DAT cells are in agreement with a recent report on COS-7 cells transfected with human DAT cDNA, in which release of dopamine was studied in 24-well plates, but no time course of efflux could be obtained with this method (25).

The larger absolute amount of dopamine released by amphetamine from DAT/VAT cells, compared with DAT cells, may be due to (i) direct mobilization of vesicular dopamine and/or (ii) exchange diffusion from a cytoplasmic pool that is, to a certain extent, fed by dopamine leaking out of the vesicles and is therefore not as easily exhausted as in DAT cells. The continuing supply of dopamine from vesicles could also explain our observation that in the presence of amphetamine the overflow of dopamine remained elevated in DAT/VAT cells but returned to base-line levels in DAT cells.

In conclusion, the presence of the plasmalemmal DAT is sufficient for amphetamine to release intracellular dopamine. However, the time course and the magnitude of the action of amphetamine are substantially modified by the presence of a vesicular amine transport system. Cocaine, which lacks a releasing action via the plasmalemmal DAT and blocks the effect of amphetamine at this site, has a dopamine-releasing action only in the presence of a SVAT.

Acknowledgments

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