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Carrier-dependent and Ca^{2+}-dependent 5-HT and dopamine release induced by (+)-amphetamine, 3,4-methylendioxymethamphetamine, *p*-chloroamphetamine and (+)-fenfluramine

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1 The mechanism underlying 5-hydroxytryptamine (5-HT) and/or dopamine release induced by (+)-amphetamine ((+)-Amph), 3,4-methylendioxymethamphetamine (MDMA), *p*-chloroamphetamine (pCA) and (+)-fenfluramine ((+)-Fen) was investigated in rat brain superfused synaptosomes preloaded with the ^3H neurotransmitters.

2 Their rank order of potency for [^{3}H]-5-HT-releasing activity was the same as for inhibition of 5-HT uptake (pCA $\geqslant$ MDMA $\geqslant$ (+)-Fen $>>$ (+)-Amph). Similarly, their rank order as [^{3}H]-dopamine releasers and dopamine uptake inhibitors was the same ((+)-Amph $>>$ pCA = MDMA $>>$ (+)-Fen). We also confirmed that the release induced by these compounds was prevented by selective transporter inhibitors (indalpine or nomifensine).

3 [^{3}H]-5-HT and/or [^{3}H]-dopamine release induced by all these compounds was partially (31–80%), but significantly Ca^{2+}-dependent. Lack of extracellular Ca^{2+} did not alter uptake mechanisms nor did it modify the carrier-dependent dopamine-induced [^{3}H]-dopamine release. (+)-Amph-induced [^{3}H]-dopamine release and pCA- and MDMA-induced [^{3}H]-5-HT release were significantly inhibited by ω-agatoxin-IVA, a specific blocker of P-type voltage-operated Ca^{2+}-channels, similar to the previous results on (+)-Fen-induced [^{3}H]-5-HT release.

4 Methiothepin inhibited the Ca^{2+}-dependent component of (+)-Amph-induced [^{3}H]-dopamine release with high potency (70 nM), as previously found with (+)-Fen-induced [^{3}H]-5-HT release. The inhibitory effect of methiothepin was not due to its effects as a transporter inhibitor or Ca^{2+}-channel blocker and is unlikely to be due to its antagonist properties on 5-$HT_{1/2}$, dopamine or any other extracellular receptor.

5 These results indicate that the release induced by these compounds is both 'carrier-mediated' and Ca^{2+}-dependent (possibly exocytotic-like), with the specific carrier allowing the amphetamines to enter the synaptosome. The Ca^{2+}-dependent release is mediated by Ca^{2+}-influx (mainly through P-type Ca^{2+}-channels), possibly triggered by the drug interacting with an unknown intracellular target, affected by methiothepin, common to both 5-HT and dopamine synaptosomes.

Keywords: (+)-Amphetamine; 3,4-methylendioxymethamphetamine; *p*-chloroamphetamine; (+)-fenfluramine; 5-hydroxytryptamine release; dopamine release; 5-hydroxytryptamine transporters; dopamine transporters; carrier-mediated release; exocytotic release

Introduction

The mechanism by which (+)-amphetamine ((+)-Amph) (Seiden *et al*., 1993) and related drugs (*p*-chloroamphetamine (pCA, Baumgarten & Zimmermann, 1992)); 3,4-methylendioxymethamphetamine (MDMA, Green *et al*., 1995) and (+)-fenfluramine ((+)-Fen, Garattini *et al*., 1992)) promote the release of catecholamines (CA) and/or 5-hydroxytryptamine (5-HT) from presynaptic nerve endings is not yet entirely clear. However, we must clarify this mechanism in order to understand its role in the pharmacological and toxicological effects of these drugs.

A 'carrier-mediated' release of the neurotransmitter (see Levi & Raiteri, 1993 for review) is widely suggested, according to the 'Exchange Diffusion Model' (Trendelemburg, 1979; Fisher & Cho, 1979; Raiteri *et al*., 1979; Liang & Rutledge, 1982; Maura *et al*., 1982). In this model amphetamine and its derivatives (called 'amphetamines' here for brevity) are transported into the nerve ending by the specific uptake carrier, although passive diffusion of these lipophilic compounds through the membrane can also occur. The carrier-mediated entry, presumably predominant at lower concentrations (Liang & Rutledge, 1982; Seiden *et al*., 1993), implies that the compounds behave as substrates of the carriers (Bonish, 1984; Zaczek *et al*., 1991; Wölfel & Graefe, 1992) and explains the inhibition of monoamine transport from outside to inside. Once inside, the amphetamines may cause neurotransmitter release from intracellular storage vesicles, either through a direct interaction with the vesicular transport or through alkalinization of the vesicles (Sulzer & Rayport, 1990; Rudnick & Wall, 1992b; Schuldiner *et al*., 1993; Pifl *et al*., 1995; Sulzer *et al*., 1995). The increase in cytoplasmic neurotransmitter (Sulzer *et al*., 1995) and/or the favourable orientation of the carrier on the inside of the nerve ending (made available by the incoming amphetamine molecules) would then promote carrier-mediated neurotransmitter release.

The concepts that the uptake carrier can, in certain conditions, transport the neurotransmitter outside the nerve ending (Raiteri *et al*., 1975; 1979; Trendelemburg, 1979; Levi & Raiteri, 1993; Sulzer *et al*., 1995) and that amphetamines may actually favour this carrier-mediated release are well established, mainly on the basis of *ad hoc* experimental models, i.e. with platelet plasma membrane vesicles (Rudnick & Wall, 1992a,b; Schuldiner *et al*., 1993), cells transfected with the transporters (Eshleman *et al*., 1994; Pifl *et al*., 1995; Wall *et al*., 1995; Cinquanta *et al*., 1997) or reserpine-treated synaptosomes (Mennini *et al*., 1981).

The involvement of the monoamine carriers in the dopamine and/or 5-HT releasing activity of (+)-Amph, MDMA, pCA and (+)-Fen is further supported by the inhibition exerted by specific carrier blockers, such as citalopram or

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fluoxetine for the 5-HT uptake carrier or nomifensine for the dopamine uptake carrier (Raiteri *et al*., 1979; Hekmatpanah & Peroutka, 1990; Berger *et al*., 1992; Gobbi *et al*., 1992; Wickems *et al*., 1995; Sulzer *et al*., 1995). However, this last result does not *per se* support the hypothesis of carrier-mediated release, since it might be due to inhibition of drug transport inside the nerve ending rather than of the outward neurotransmitter transport.

Another experimental characteristic presumably associated with carrier-mediated release, differentiating it from depolarization-induced exocytotic release, is Ca^{2+}-independency (Levi & Raiteri, 1993). *In vitro* data are controversial, since amphetamines-induced release was found to be completely Ca^{2+}-independent in some studies (Takimoto *et al*., 1983; Johnson *et al*., 1986; Wickems *et al*., 1995), but partially Ca^{2+}-dependent in others (Bowyer *et al*., 1984; Gobbi *et al*., 1992; 1993; Bonanno *et al*., 1994). These differences may be due to the experimental models employed (rat brain synaptosomes versus brain slices or cultured foetal rat neurones; superfusion versus incubation, see below). We and others (Gobbi *et al*., 1992; Bonanno *et al*., 1994) described a Ca^{2+}-dependent [^{3}H]-5-HT release induced by low (but not high) (+)-Fen concentrations from rat and human brain synaptosomes. Further investigation suggested that (+)-Fen, after its carrier-mediated entry into the 5-HT nerve endings, might induce an influx of Ca^{2+}-ions, triggering an exocytotic-like release of 5-HT (Gobbi *et al*., 1993; Frittoli *et al*., 1994). Thus, other mechanisms, different from the carrier-mediated one, may underlie at least part of the amphetamines-induced neurotransmitter release.

We therefore decided to re-evaluate the 5-HT and/or dopamine releasing activity of (+)-Amph, pCA, MDMA and (+)-Fen, in the same experimental model, i.e. superfused rat brain synaptosomes preloaded with the ^{3}H neurotransmitter. Using synaptosomes from native brain tissue should enable us to study release mechanisms potentially relevant *in vivo*; it is also clear that synaptosomes, i.e. intact nerve endings with the complete exocytotic machinery, are the best structures for detecting the suggested exocytotic-like release. It should also be noted that with the superfusion apparatus employed (Raiteri *et al*., 1974), the neurotransmitter released by the drug is immediately removed and cannot interact with presynaptic receptors or be retaken up (Raiteri *et al*., 1979; Maura *et al*., 1982; Gobbi *et al*., 1992). Thus, the releasing effect is completely distinct from uptake inhibition, whereas with other 'static' experimental models (i.e. release in test tubes or wells) the measured releasing effect may also be due to reuptake inhibition (Seiden *et al*., 1993). This is particularly important when comparing the effects of compounds that act both as releasers and reuptake inhibitors.

We first established the rank order of potency of these compounds as 5-HT and/or dopamine releasers and as 5-HT and/or dopamine uptake inhibitors, in order to clarify the transporters' role in the releasing activity and their selectivity. We also investigated the carrier- and Ca^{2+}-dependency of their releasing effects, in order to cast light on the molecular mechanism and on any differences between the compounds tested.

Methods

Preparation of the synaptosomal fraction

Male CRL:CD(SD)BR rats (Charles River, Italy), weighing about 150 g, were used. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L.n. 116, G.U., suppl. 40, Feb. 18, 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council 1996).

The rats were killed by decapitation and their hippocampi and striata were rapidly dissected and homogenized in 40 vol of ice-chilled 0.32 M sucrose, pH 7.4, in a glass homogenizer with a Teflon pestle. The homogenates were centrifuged at $1000 \times g$ for 5 min and the supernatants centrifuged again at $12{,}000 \times g$ for 20 min to yield the crude synaptosomal pellet (P_2) (Gray & Whittaker, 1962).

Uptake studies

The P_2 pellets were diluted to a concentration of about 5 mg wet weight tissue ml^{-1} with Krebs-Henseleit buffer having the following composition (mM): NaCl 116, $NaHCO_3$ 25, NaH_2PO_4 1, KCl 6, $MgSO_4$ 1, $CaCl_2$ 2, glucose 10, pargyline 0.25, EDTA 0.07 and ascorbic acid 0.3, pH 7.2–7.4. When required, a Ca^{2+}-free medium was used in which $CaCl_2$ was replaced with 3 mM EGTA for [^{3}H]-5-HT uptake or with 0.03 mM EGTA for [^{3}H]-dopamine uptake. Samples of 0.6 ml were preincubated for 5 min at 30°C in a water bath with or without the compounds to be tested. Non specific uptake was determined in the presence of 0.3 μM citalopram ([^{3}H]-5-HT uptake) or 1 μM nomifensine ([^{3}H]-dopamine uptake).

Uptake was started by the addition of 30 nM [^{3}H]-dopamine (NEN, Dreiech, Germany; S.A. 11.8 Ci $mmol^{-1}$) or [^{3}H]-5-HT (Amersham, Buckinghamshire, U.K.; S.A. 29.7 Ci $mmol^{-1}$) and the reaction was stopped 5 min later by adding 1 ml of ice-chilled Krebs-Henseleit buffer. Samples were immediately filtered through cellulose mixed esters filters (0.65 μm pore size, Millipore) and washed twice with 2 ml of Krebs-Henseleit buffer. The radioactivity trapped on the filters was counted in 4 ml of Ultima Gold MV (Packard) in a LKB 1214 Rackbeta liquid scintillation counter with a counting efficiency of about 60%.

Dose-inhibition curves were analysed by the 'Allfit' programme running on an IBM-AT personal computer (DeLean *et al*., 1978).

Release studies

The P_2 pellets were resuspended in about 20 vol Krebs-Henseleit buffer with the following composition (mM): NaCl 125, KCl 3, $CaCl_2$ 1.2, $MgSO_4$ 1.2, NaH_2PO_4 1, $NaHCO_3$ 22 and glucose 10; gassed with 95% O_2 and 5% CO_2, pH 7.2–7.4. The suspension was then added to an equal volume of the same buffer containing [^{3}H]-5-HT (Amersham, Buckinghamshire, U.K.; S.A. 29.7 Ci $mmol^{-1}$) or [^{3}H]-dopamine (NEN, Dreiech, Germany; S.A. 11.8 Ci $mmol^{-1}$), both at the final concentration of 0.06 μM. After 15 min incubation at 37°C, the suspension was diluted with fresh buffer and 5 ml samples (about 5 mg initial tissue) were distributed onto cellulose mixed esters filters (0.65 μm pore size, Millipore) in a 16-chamber superfusion apparatus held thermostatically at 37°C (Raiteri *et al*., 1974).

The synaptosomes were layered onto the filters by aspiration from the bottom under moderate vacuum. Superfusion was started ($t = 0$ min) at a rate of 0.5 ml min^{-1} with standard medium; after 42 min equilibration period, fractions were collected every 2 min until $t = 60$ min. The filters were put into scintillation vials and counted for radioactivity, as the fractions, in 4 ml of Ultima Gold MV (Packard).

(+)-Amph, pCA and MDMA (RBI, U.S.A.) were present in the superfusion medium for 3 min from $t = 47$, to $t = 50$ min; similarly, the synaptosomes were depolarized by replacing the normal medium from $t = 47$ to $t = 50$ with one containing a higher KCl concentration (15 mM instead of an equimolar concentration of NaCl). When used, 1 μM indalpine (Rhone-Poulenc, France) or 3 μM nomifensine (RBI, U.S.A.) were present from $t = 40$ to $t = 60$ min for [^{3}H]-5-HT release or [^{3}H]-dopamine release, respectively. In some experiments, synaptosomes were superfused from $t = 40$ to $t = 60$ min with a Ca^{2+}-free medium containing 3 mM EGTA for [^{3}H]-5-HT release or 0.03 mM EGTA for [^{3}H]-dopamine release (in these conditions

there was no effect on basal release whereas the depolarization-induced release was completely blocked). When used, ω-agatoxin-IVA (Alexis Co., Switzerland) was added to the superfusion medium from $t=40$ to $t=50$. Methiothepin (RBI, U.S.A.) was added to the superfusion medium from $t=40$ to $t=50$.

The fractional release rate (FRR) was calculated as 100 times the amount of radioactivity released into each 2-min fraction over the total radioactivity present on the filter at the start of that fraction. The FRRs found before the releasing stimulus ($t=44-46$), expressed as percentage in 2 min, are presented as basal outflow. The overflow (%) was calculated as the difference between the FRR in the presence ($t=48-56$) and absence of the drug (mean $t=44-48$ and $t=56-60$). The effect of the drugs added at $t=47$ was only detectable 1 min later, since the fluid takes about 1.5 min to flow from the filters to the collecting vials.

The content of unmetabolized [^{3}H]-5-HT or [^{3}H]-dopamine in the superfusate was determined as previously described (Goldstein *et al*., 1981; Gobbi *et al*., 1992).

Results

Potency of amphetamines as uptake inhibitors

The IC_{50} values for (+)-Fen, pCA, MDMA and (+)-Amph for the inhibition of [^{3}H]-5-HT and [^{3}H]-dopamine uptake in rat hippocampal and striatal synaptosomes are shown in Table 1. The rank order of potency on [^{3}H]-5-HT uptake was: MDMA = pCA ≥ (+)-Fen > (+)-Amph, and on [^{3}H]-dopamine uptake was: (+)-Amph > pCA = MDMA > (+)-Fen. As regards the selectivity, (+)-Fen was 12.4 times more potent on [^{3}H]-5-HT uptake than on [^{3}H]-dopamine uptake, whereas (+)-Amph was 34.7 times more potent on [^{3}H]-dopamine uptake than on [^{3}H]-5-HT uptake. pCA and MDMA were only slightly more potent on [^{3}H]-5-HT than on [^{3}H]-dopamine uptake (1.7 and 3.2 times, respectively).

The potency of these compounds for the inhibition of [^{3}H]-5-HT and [^{3}H]-dopamine uptake, and the specific ^{3}H neurotransmitter uptake in the absence of drugs (see legend of Table 1), was independent of the presence of Ca^{2+} ions in the incubation medium (Table 1).

Potency of amphetamines as releasers

Figure 1 shows the concentration-response curves for the releasing effects of (+)-Fen, pCA, MDMA and (+)-Amph when applied for 3 min to superfused synaptosomes preloaded with [^{3}H]-5-HT (hippocampal synaptosomes) or [^{3}H]-dopamine (striatal synaptosomes). For purposes of comparison, Figure 1 also shows the overflow induced by 15 mM K^+ (i.e. by depolarization) which, in the same experimental conditions, amounted to 10.8 ± 0.7 ($n=6$) for [^{3}H]-5-HT and to 9.1 ± 2.4 ($n=9$) for [^{3}H]-dopamine (indicated as dashed lines).

The tritium overflow evoked by the releasing agents tested was mainly unmetabolized ^{3}H neurotransmitter. [^{3}H]-5-HT amounted to 95 ± 7%, 82 ± 6% and 75 ± 9% of the tritium

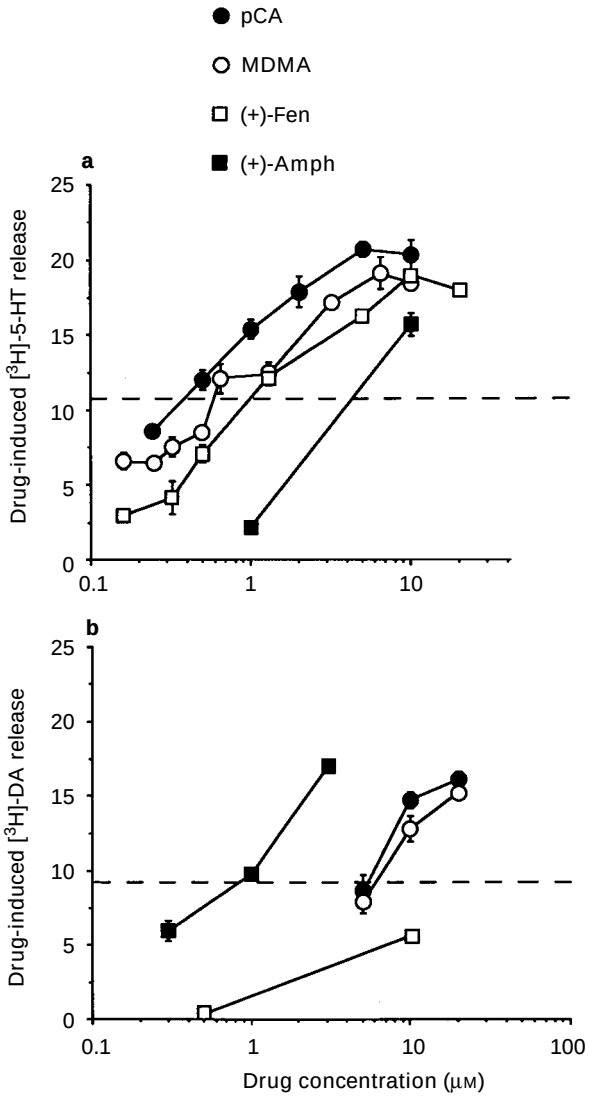

Figure 1 Concentration-effect curves for *p*-chloroamphetamine (pCA), 3,4-methylendioxymethamphetamine (MDMA), (+)-fenfluramine ((+)-Fen) and (+)-amphetamine ((+)-Amph) induced [^{3}H]-5-HT release from rat hippocampal synaptosomes (a) or [^{3}H]-dopamine ([^{3}H]-DA) release from rat striatal synaptosomes (b). Superfused synaptosomes were exposed to the releasing agent for 3 min. The release induced by depolarization (15 mM K^+) is shown by the dashed lines, for comparison. Each overflow value is the mean of 3–5 results from 1–2 experiments; vertical lines show s.d.

Table 1 Inhibitory activity of (+)-fenfluramine, *p*-chloroamphetamine (pCA), 3,4-methylendioxymethamphetamine (MDMA) and (+)-amphetamine on [^{3}H]-5-HT uptake in rat hippocampal synaptosomes or on [^{3}H]-dopamine (DA) uptake in rat striatal synaptosomes

	IC_{50} (μM)			
	[^{3}H]-5-HT uptake		*[^{3}H]-DA uptake*	
	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$
(+)-Fenfluramine	0.90 ± 0.40	0.70 ± 0.20	11.2 ± 1.3	9.8 ± 0.9
pCA	0.53 ± 0.16	0.45 ± 0.18	0.90 ± 0.07	0.80 ± 0.10
MDMA	0.35 ± 0.03	0.35 ± 0.03	1.14 ± 0.03	1.14 ± 0.10
(+)-Amphetamine	4.51 ± 0.64	4.11 ± 0.37	0.13 ± 0.04	0.15 ± 0.01

The uptake was measured with 30 nM of the ^{3}H neurotransmitter, either with or without 1.2 mM $CaCl_2$ in the incubation medium. Specific [^{3}H]-5-HT uptake with and without Ca^{2+} ions was 12.77 and 15.3 fmol min^{-1} mg^{-1} tissue, respectively. Specific [^{3}H]-DA uptake with and without Ca^{2+} ions was 237 and 266 fmol min^{-1} mg^{-1} tissue. Five different concentrations of each compound were used in triplicate, and the inhibition curves were analysed according to the logistic function (DeLean *et al*., 1978) giving the IC_{50} values ± s.e.

overflow evoked by 0.5, 10 and 20 μM (+)-Fen (means ± s.d., $n=3$), confirming previous data (Gobbi *et al.*, 1992); it was 97 ± 5%, 93 ± 2% and 92 ± 0.3% of the tritium overflow evoked by 0.25, 0.5 and 5 μM PCA ($n=3$) and 81 ± 13%, 81 ± 12% and 73 ± 8% of the tritium overflow evoked by 0.25, 0.5 and 5 μM MDMA ($n=3$). Similarly, unmetabolized [^{3}H]-dopamine amounted to 94 ± 7%, 77 ± 14% and 83 ± 15% of the tritium overflow evoked by 1 μM (+)-Amph, 5 μM MDMA and 5 μM pCA ($n=4$). Thus, the tritium overflow induced by (+)-Fen, pCA, MDMA and (+)-Amph will be referred to as [^{3}H]-5-HT or [^{3}H]-dopamine release.

The rank order of potency for inducing [^{3}H]-5-HT release was pCA ≥ MDMA ≥ (+)-Fen > > (+)-Amph. The concentrations (μM) giving the same overflow as that due to 15 mM K^+ were 0.4 for pCA, 0.6 for MDMA, 1.0 for (+)-Fen and 4.2 for (+)-Amph. The rank order of potency in inducing [^{3}H]-dopamine release was (+)-Amph > > pCA = MDMA > > (+)-Fen. The concentrations (μM) giving the same overflow as that due to 15 mM K^+ were 0.9 for (+)-Amph, 5–6 for pCA and MDMA and > 10 for (+)-Fen. Thus (+)-Fen, pCA and MDMA were more potent in inducing [^{3}H]-5-HT release than [^{3}H]-dopamine release, whereas the opposite was true for (+)-Amph.

We subsequently characterized the [^{3}H]-5-HT releasing effect of 0.5 μM pCA and MDMA (comparing them with the effect of 0.5 μM (+)-Fen); and the [^{3}H]-dopamine releasing effect of 1 μM (+)-Amph and 5 μM pCA and MDMA.

Carrier- and Ca^{2+}-dependence of the drug-induced release

Indalpine and nomifensine, at concentrations up to 10 μmol l^{-1}, had no effect on basal [^{3}H]-5-HT and [^{3}H]-dopamine release (data not shown), confirming that, with our superfusion technique, a pure re-uptake blocker has no detectable releasing effect. The [^{3}H]-5-HT overflow induced by 0.5 μM (+)-Fen, pCA and MDMA was completely abolished in the presence of 1 μM indalpine ((+)-Fen from 7.1 ± 0.6 to 0.5 ± 0.4; pCA from 12.0 ± 0.6 to 0.1 ± 0.2; MDMA from 8.5 ± 0.4 to 0.1 ± 0.3). The [^{3}H]-dopamine overflow induced by 1 μM (+)-Amph or by 5 μM pCA and MDMA was completely abolished in the presence of 3 μM nomifensine ((+)-Amph from 10.7 ± 0.4 to 0.6 ± 0.4; pCA from 8.8 ± 0.6 to 1.2 ± 0.4; MDMA from 9.7 ± 0.1 to 1.1 ± 0.4).

The removal of Ca^{2+} ions from the superfusion buffer (with addition of 3 mM EGTA for [^{3}H]-5-HT or 0.03 mM EGTA for [^{3}H]-dopamine release) had no significant effect on basal tritium efflux (see legends of Figures 2 and 3) whereas it completely abolished the depolarization-induced release ((Gobbi *et al.*, 1992) and data not shown).

The removal of extracellular Ca^{2+} ions partially but significantly reduced [^{3}H]-5-HT release induced by PCA and MDMA (Figure 2). [^{3}H]-5-HT overflow induced by 0.25, 0.5, 5 and 10 μM PCA was reduced by 67%, 64%, 44% and 45%, respectively, and [^{3}H]-5-HT overflow induced by 0.25, 0.5, 5 and 10 μM MDMA was reduced by about 68%, 64%, 49% and 58%.

Figure 3 shows that the removal of extracellular Ca^{2+} ions from the superfusion buffer also partially but significantly reduced [^{3}H]-dopamine release induced by 1 μM (+)-Amph (by 55%), by 5 and 20 μM PCA (by 43% and 31%) and by 5 and 20 μM MDMA (by 52% and 31%).

As a positive control for Ca^{2+}-independent, carrier-mediated [^{3}H]-dopamine release, we investigated the release induced by dopamine itself, measured in the presence of 250 μM pargyline. This release was completely abolished in the presence of 3 μM nomifensine but was not affected by the removal of Ca^{2+}-ions from the superfusion buffer: with 30 nM dopamine, the overflow was respectively 4.2 ± 0.5 and 4.9 ± 0.8 with and without Ca^{2+}-ions; with 100 nM dopamine, the overflow was 19.3 ± 0.1 with and 21.9 ± 0.8 without Ca^{2+} ions; with 1000 nM dopamine, the overflow was 68.0 ± 4.6 with and 68.7 ± 7.6 without Ca^{2+} ions (means ± s.d., $n=3$).

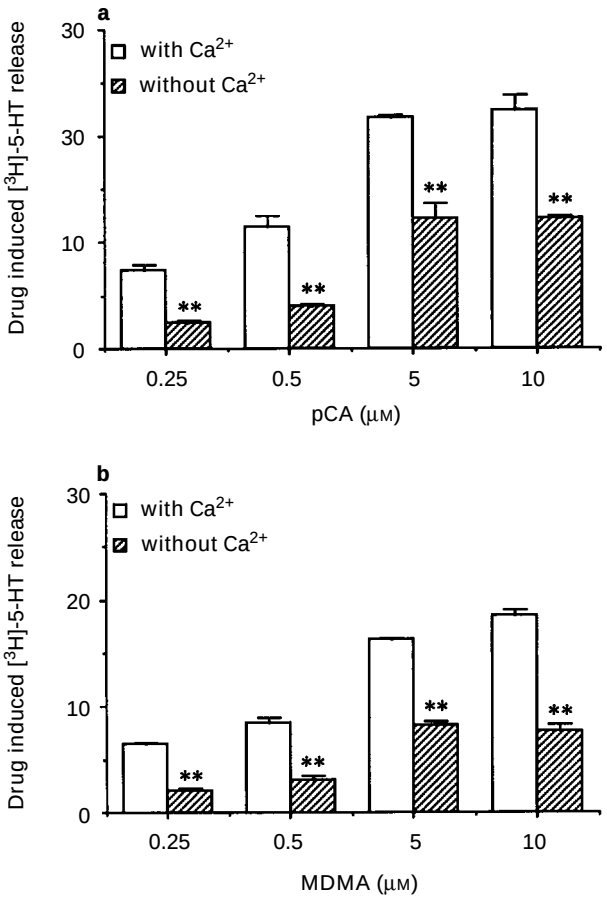

Figure 2 Effect of extracellular Ca^{2+} ions on *p*-chloroamphetamine- (pCA) and 3,4-methylendioxymethamphetamine- (MDMA) induced [^{3}H]-5-HT release from rat superfused hippocampal synaptosomes. The releasing stimuli were applied for 3 min. The effect of extracellular Ca^{2+} was evaluated by use of a Ca^{2+}-free medium containing 3 mM EGTA; the normal medium contained 1.2 mM $CaCl_2$. Basal outflow (as the percentage in 2 min ± s.d.) was 1.82 ± 0.23 with Ca^{2+} and 1.52 ± 0.32 without. Each value is the mean ± s.d. of 3–4 results. *$P<0.05$; **$P<0.01$ (Student's *t* test).

Figure 4 shows that 100 nM ω-agatoxin IVA, a blocker of the P-type voltage-operated Ca^{2+}-channels, partially but significantly counteracted (+)-Amph-induced [^{3}H]-dopamine release and pCA- or MDMA-induced [^{3}H]-5-HT release, similar to its effect on (+)-Fen-induced [^{3}H]-5-HT release (Frittoli *et al.*, 1994) and on depolarization-induced [^{3}H]-dopamine (Figure 4a) and [^{3}H]-5-HT release (Figure 4b).

Effect of methiothepin on (+)-Amph-induced [^{3}H]-dopamine release

We recently showed that [^{3}H]-5-HT release induced by 0.5 μM (+)-Fen from rat hippocampal synaptosomes was inhibited with high affinity by methiothepin (Mennini *et al.*, 1996; see Discussion). In the present study we therefore tested the effect of methiothepin on (+)-Amph-induced [^{3}H]-dopamine release in order to assess its specificity. At the concentrations used methiothepin did not affect basal [^{3}H]-dopamine release (not shown).

Methiothepin dose-dependently inhibited [^{3}H]-dopamine overflow induced by 1 μM (+)-Amph (Figure 5a): non-linear fitting of the data according to the logistic function (DeLean *et al.*, 1978) indicated that inhibition was only partial even at high methiothepin concentrations, with 60% inhibition at the highest concentration tested, and an IC_{50} concentration causing half-maximal inhibition, calculated to be about 70 nM. Figure 5b shows that the inhibitory effect of 300 nM methiothepin was apparent only in the presence of Ca^{2+} ions in the

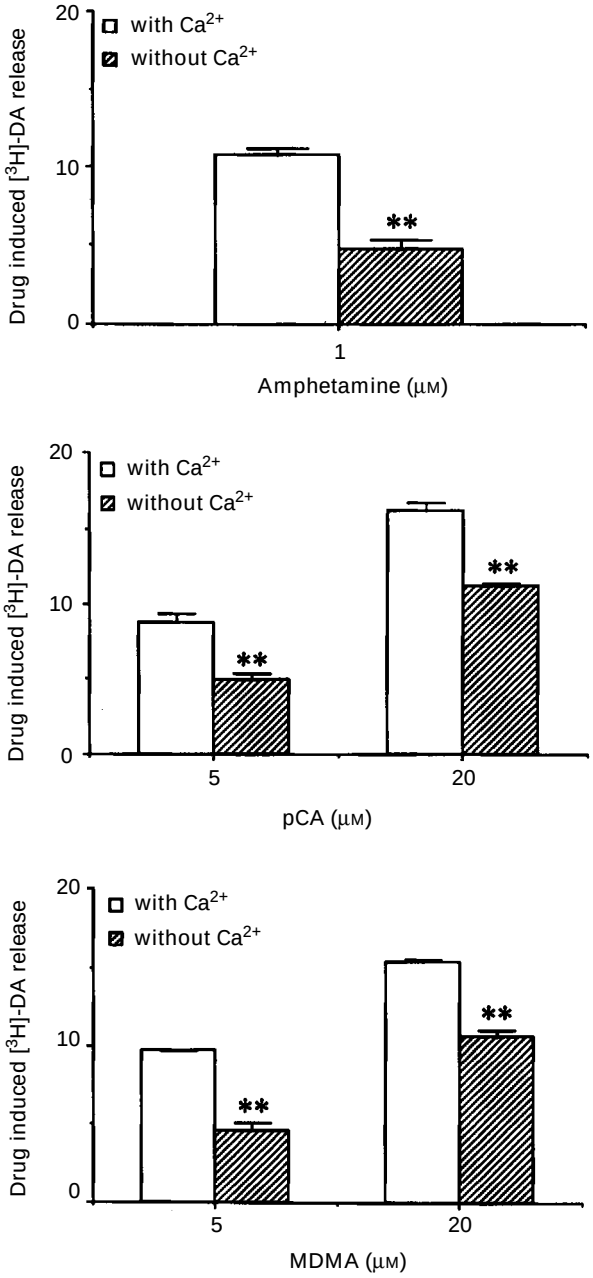

Figure 3 Effect of extracellular Ca^{2+} ions on (+)-amphetamine ((+)-Amph)- *p*-chloroamphetamine (pCA)- and 3,4-methylendioxymethamphetamine (MDMA)-induced [^{3}H]-dopamine ([^{3}H]-DA) release from rat superfused striatal synaptosomes. The releasing stimuli were applied for 3 min. The effect of extracellular Ca^{2+} was evaluated by use of a Ca^{2+}-free medium containing 0.03 mM EGTA; the normal medium contained 1.2 mM $CaCl_2$. Basal outflow (as the percentage in 2 min ± s.d.) was 2.20 ± 0.45 with Ca^{2+} and 1.92 ± 0.36 without. Each overflow value is the mean ± s.d. of 3–4 results. $^*P<0.05$; $^{**}P<0.01$ (Student's *t* test).

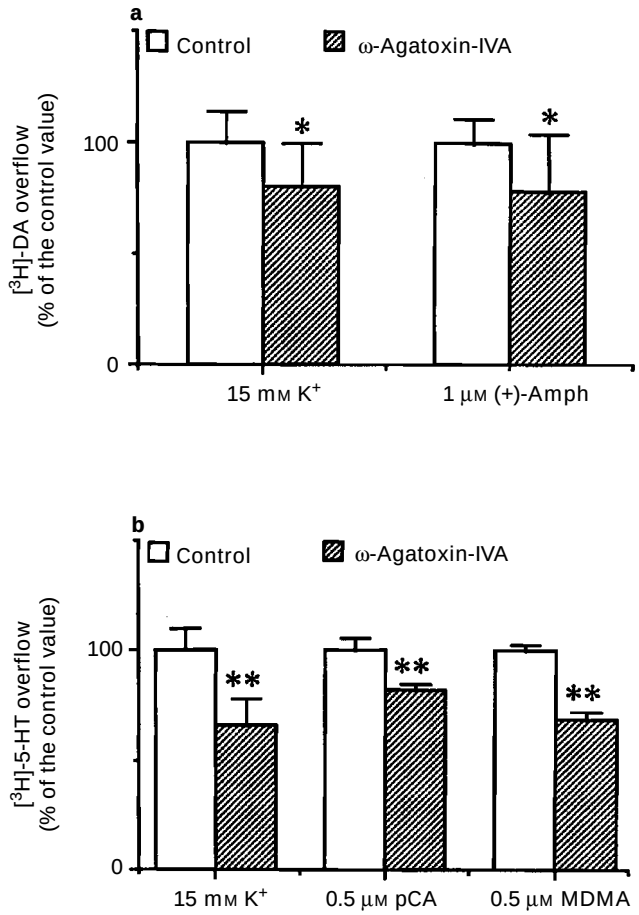

Figure 4 Effect of ω-agatoxin-IVA (100 nM) on drug- and depolarization-induced [^{3}H]-dopamine ([^{3}H]-DA) release from rat superfused striated synaptosomes (a) and [^{3}H]-5-HT release from rat superfused hippocampal synaptosomes (b). The synaptosomes were exposed to the releasing agent for 3 min (from $t=47$ to $t=50$) in the absence or presence of ω-agatoxin-IVA (added to the superfusion medium from $t=40$ to $t=50$). Basal outflow (as the percentage in 2 min ± s.d.) in the absence and presence of ω-agatoxin-IVA was 2.36 ± 0.15 and 2.14 ± 0.31 for [^{3}H]-DA release and 1.63 ± 0.06 and 1.57 ± 0.01 for [^{3}H]-5-HT release. Each value is the mean ± s.d. of 3–5 results from 1–2 experiments and represents the overflow in the presence of ω-agatoxin-IVA as a percentage of that without it. $^*P<0.05$; $^*P<0.01$ (Student's *t* test).

superfusion buffer, thus suggesting it is specific for the Ca^{2+}-dependent component of the (+)-Amph-induced [^{3}H]-dopamine release.

We verified that methiothepin did not affect [^{3}H]-dopamine uptake into synaptosomes (240 ± 5 and 252 ± 5 fmol min^{-1} mg^{-1} tissue, in the absence and presence of 300 nM methiothepin, respectively). The potency of (+)-Amph for inhibition of synaptosomal [^{3}H]-dopamine uptake was also not affected, or it was even slightly increased, by methiothepin (IC_{50} values: 0.14 ± 0.01 and 0.10 ± 0.1 nM, in the absence and presence of 300 nM methiothepin, respectively). Moreover, 300 nM methiothepin did not affect K^+-induced [^{3}H]-dopamine release (the overflow being 12.93 ± 0.96 in the absence and 12.83 ± 0.51 nM in the presence of 300 nM methiothepin), similar to K^+-induced [^{3}H]-5-HT release (Mennini *et al.*, 1996).

Discussion

Releasing properties of amphetamines and involvement of the transporter protein

The rank order of potency of (+)-Amph and its derivatives as 5-HT releasers and as 5-HT uptake inhibitors was the same (pCA ≥ MDMA ≥ (+)-Fen > (+)-Amph); the drug concentrations giving the same release as that induced by depolarization were comparable to the IC_{50} for [^{3}H]-5-HT uptake (about 0.4 μM for pCA and MDMA, 0.8 μM for (+)-Fen and 4 μM for (+)-Amph).

These data are in agreement with previous findings (McKenna *et al.*, 1991; Berger *et al.*, 1992), showing a similar 5-HT releasing potency for pCA, (+)-Fen and MDMA in rat brain synaptosomes, while in cultured foetal rat neurones pCA was ten times as potent as the other two compounds (Wickems *et al.*, 1995).

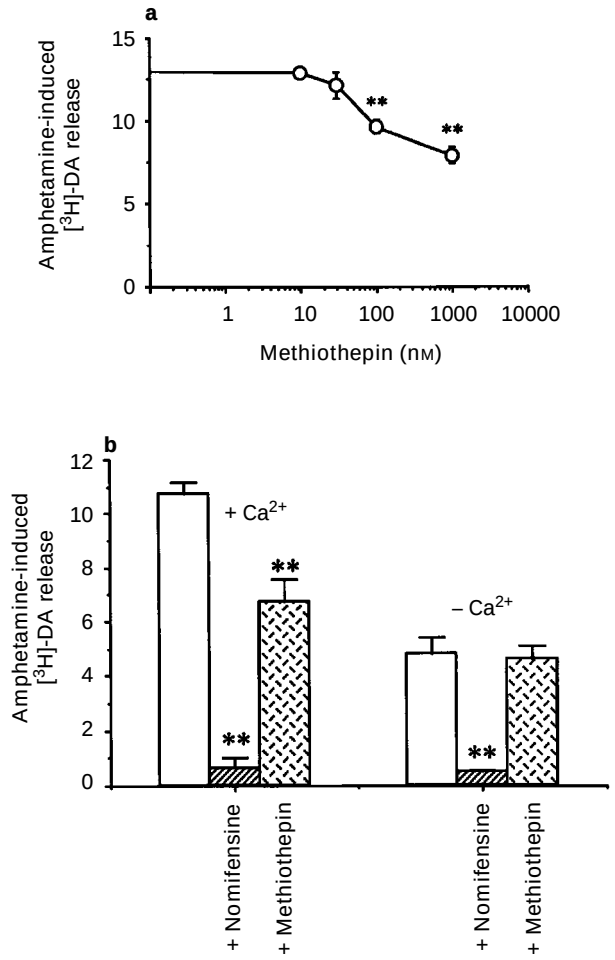

Figure 5 Effect of methiothepin on (+)-amphetamine-induced [^{3}H]-dopamine ([^{3}H]-DA) release from rat superfused striatal synaptosomes. The synaptosomes were exposed to 1 μM (+)-amphetamine for 3 min (from $t=47$ to $t=50$) in the absence and presence of methiothepin (added to the superfusion medium from $t=40$ to $t=50$). (a) Concentration-response curve; (b) effect of 300 nM methiothepin (and 3 μM nomifensine) in the presence or absence of 1.2 mM $CaCl_2$ in the superfusion medium (with 0.03 mM EGTA in the latter condition). Each overflow value is the mean ± s.d. of 3–5 results. $^*P<0.05$; $^{**}P<0.01$ (Student's t test).

The rank order of potency for dopamine-releasing activity was also very similar to that for inhibition of dopamine uptake ((+)-Amph > pCA = MDMA > (+)-Fen); in this case, the drug concentrations giving the same release as that induced by depolarization (about 0.8 μM for (+)-Amph, 5 μM for pCA and MDMA and > 10 μM for (+)-Fen) were about five times the IC_{50} for [^{3}H]-dopamine uptake (about 0.13 μM for (+)-Amph, 1 μM for pCA and MDMA and 11 μM for (+)-Fen).

As discussed in the introduction, the superfusion technique used for the release assay avoids the possibility of the potency of the amphetamines as releasers being confounded by their effects as reuptake inhibitors. This is clearly confirmed by the fact that indalpine (a selective 5-HT reuptake blocker) and nomifensine (a selective dopamine reuptake blocker) had no [^{3}H]-5-HT or [^{3}H]-dopamine releasing activity *per se*. On the other hand, the possibility that the inhibitory activities of amphetamines measured in the ^{3}H monoamine uptake experiments may partly result from the concomitant release induced by these compounds cannot be excluded (Levi & Raiteri, 1974; Seiden *et al*., 1993). Accordingly, the potency of (+)-Fen as inhibitor of [^{3}H]-paroxetine binding is lower than its potency on [^{3}H]-5-HT uptake (Mennini *et al*., 1991); similarly, (+)-Amph is less potent on [^{3}H]-GBR-12783 than on [^{3}H]-dopamine uptake (Bonnet *et al*., 1986).

The most likely explanation of our results, in agreement with the current view, is that amphetamines interact with the transporter protein as competing substrates (Bonish, 1984; Wölfel & Graefe, 1992), and that the releasing properties of these compounds involve their transport by the specific carrier into the nerve ending. This is the only explanation for the selectivity of the 'amphetamines' for the 5-HT or the dopamine system: (+)-Amph has a greater affinity (35 fold) for the dopamine transporter than for the 5-HT transporter and is a rather selective dopamine releaser; the reverse is true for (+)-Fen, which has a 12 times greater selectivity for the 5-HT transporter and behaves as a preferential 5-HT releaser. If the amphetamines entered the nerve ending only (or mainly) by diffusion, the selectivity would remain unexplained, except for the possibility of a different interaction with the dopamine- or 5-HT-storage vesicles, but this has no experimental validation. Carrier-mediated entry of the drug into the synaptosomes would also explain the sensitivity to carrier blockers (indalpine for 5-HT release and nomifensine for dopamine release), although these compounds may also inhibit carrier-mediated release of the neurotransmitter.

Ca^{2+} dependence of the amphetamine-induced release

In the second part of the study we investigated the Ca^{2+}-independence of the amphetamine-induced release, since this is a characteristic of carrier-mediated release (Levi & Raiteri, 1993). We confirmed that dopamine-induced [^{3}H]-dopamine release, expected to be only due to carrier-mediated release (Liang & Rutledge, 1982; Bowyer *et al*., 1984), was completely insensitive to extracellular Ca^{2+} ions, but was completely inhibited by blocking the dopamine carrier with nomifensine. On the other hand, the [^{3}H]-5-HT or [^{3}H]-dopamine release induced by all the amphetamines we tested was partially, but significantly, Ca^{2+}-dependent, thus confirming our previous findings with (+)-Fen (Gobbi *et al*., 1992; 1993). The Ca^{2+}-dependent release ranged from 31 to 80% of the total drug-induced release, depending on the drugs and their concentrations. It seems a general rule that the lower concentrations show greater Ca^{2+} dependence, although the differences were not as marked as those for (+)-Fen-induced [^{3}H]-5-HT release, which was 82% Ca^{2+}-dependent with 0.5 μM (+)-Fen and 30% Ca^{2+}-dependent with 10 μM (+)-Fen (Gobbi *et al*., 1992).

It was recently shown that [^{3}H]-5-HT and [^{3}H]-dopamine uptake in rat brain synaptosomes depends on the presence of extracellular Ca^{2+} ions (Uchikawa *et al*., 1995; Yura *et al*., 1996), thus possibly explaining the Ca^{2+}-dependence we found for amphetamine-induced release. However, in our experimental conditions [^{3}H]-5-HT and [^{3}H]-dopamine uptake were not modified by removing Ca^{2+} ions from the buffer, either using the Krebs medium described here for uptake experiments (see legend to Table 1) or that described for release studies ((Gobbi *et al*., 1993) and data not shown). We then found that [^{3}H]-5-HT and [^{3}H]-dopamine uptake were decreased in a Ca^{2+}-free Krebs only when the medium was also depleted of Mg^{2+} (data not shown), which were the conditions used by Uchikawa *et al*. (1995) and Yura *et al*. (1996); such a finding had already been documented (White, 1975). More importantly, amphetamines gave the same IC_{50} for 5-HT or dopamine uptake carriers in the presence and absence of Ca^{2+} ions, thus excluding the possibility that the Ca^{2+} dependence of the releasing effect is due to an altered interaction of the amphetamines with the uptake carriers. The fact that Ca^{2+} ions are required for a further step in the release mechanism is also confirmed by the finding that (+)-Amph-induced [^{3}H]-dopamine release and pCA- and MDMA-induced [^{3}H]-5-HT release were significantly inhibited by ω-agatoxin-IVA, a specific blocker of P-type voltage-operated Ca^{2+} channels (Mintz *et al*., 1992), confirming similar data for (+)-Fen-induced [^{3}H]-5-HT release (Frittoli *et al*., 1994). This indicates that Ca^{2+}-dependent release is mediated by Ca^{2+} entry into the synaptosome through the voltage-operated Ca^{2+} channels and we

suggest that the increase in intracellular Ca^{2+} ions may trigger an exocytotic release. This possibility was demonstrated for (+)-Fen-induced [^{3}H]-5-HT release by the inhibitory effect of tetanus toxin, which affects the final steps of the exocytotic process (Gobbi *et al*., 1993).

It is also worth noting that indalpine or nomifensine completely inhibited amphetamine-induced release. If this release is not carrier-mediated but exocytotic-like, it follows that indalpine or nomifensine block the carrier-mediated entry of amphetamines into the synaptosome.

Effect of methiothepin on the Ca^{2+}-dependent release: implications for the possible mechanism

To investigate the possible target of the amphetamines for the induction of Ca^{2+}-dependent release we tested the effect of methiothepin. We had previously found that methiothepin dose-dependently inhibited [^{3}H]-5-HT release induced by 0.5 but not 10 μM (+)-Fen, suggesting that methiothepin inhibits only the Ca^{2+}-dependent release with high affinity (36 nM) (Mennini *et al*., 1996).

When we tested the effects of methiothepin on (+)-Amph-induced [^{3}H]-dopamine release, we confirmed a high-affinity inhibitory effect (70 nM) not due to inhibition of the dopamine carrier and specific for the Ca^{2+}-dependent component. However, methiothepin did not inhibit the Ca^{2+}-dependent [^{3}H]-dopamine release induced by depolarization, or depolarization-induced Ca^{2+}-dependent [^{3}H]-5-HT release (Mennini *et al*., 1996).

This finding has many implications: (1) methiothepin is a tool which distinguishes the Ca^{2+}-dependent and independent components of the release, thus supporting the notion of two completely distinct mechanisms; (2) methiothepin inhibits the Ca^{2+}-dependent component without acting on the voltage-operated Ca^{2+} channels or on the subsequent exocytotic process (as shown by the lack of effect on depolarization-induced release). Thus, methiothepin seems to act upstream of the (+)-Amph- or (+)-Fen-induced Ca^{2+} influx through voltage-operated Ca^{2+} channels; (3) methiothepin antagonizes the Ca^{2+}-dependent release without affecting the transporter function, thus indicating that it acts downstream of the amphetamines entry into the terminal. Recent data support the view that neurotransmitter transporters generate electrical signals which may be of sufficient magnitude to open voltage-dependent Ca^{2+} channels (Mager *et al*., 1995; Galli *et al*., 1996; Sonders & Amara, 1996). However, our data with methiothepin apparently exclude the possibility that the Ca^{2+}-dependent release induced by the amphetamines was simply due to an increase in transmitter-mediated ionic currents. Therefore our data on native tissues do not indicate that the substrate-induced transporters 'activation' results in membrane depolarization sufficient to induce neurotransmitter release, as also suggested by the Ca^{2+}-independent, carrier-mediated release induced by dopamine. (4) If methiothepin does not either alter the transporter function or interact with the mechanisms downstream of the Ca^{2+}-influx leading to exocytosis, it follows that the amphetamines-induced Ca^{2+}-influx (and the following release) must involve another step which is antagonized by methiothepin. This step is very probably intracellular, otherwise the inhibitory effects of uptake blockers would remain unexplained, and is common to both 5-HT and dopamine synaptosomes. The intracellular target of amphetamines could be, for example, a protein-kinase that phosphorylates, or indirectly activates, the Ca^{2+} channels or a K^+ channel responsible for membrane depolarization (Premkumar & Ahern, 1995). (5) The inhibitory effect of methiothepin cannot be explained by its antagonist properties on 5-$HT_{1/2}$ receptors, dopamine receptors (Hoyer, 1988) or any other extracellular receptor, unless the amphetamines activate the same receptors to induce the release. This is unlikely since the amphetamines-induced release could be inhibited by selective uptake blockers. Our experimental model (i.e. superfused synaptosomes) also precludes the possibility that the neurotransmitter released, which is immediately removed, interacts with presynaptic auto- or hetero-receptors, as shown by the lack of effect of methiothepin on the K^+-induced [^{3}H]-5-HT release (Mennini *et al*., 1996). The most likely explanation is that methiothepin interacts directly, as an antagonist, with the intracellular target on which (+)-Amph and (+)-Fen act as agonists. (6) Finally, methiothepin appeared to prevent the long lasting indole depletion induced by high (+)-Fen doses *in vivo* (Gardier *et al*., 1992), suggesting a possible role of Ca^{2+}-dependent release. Flunarizine is another compound which may interact with the same target, as it reduces (+)-Fen-induced [^{3}H]-5-HT release and the long-term indole depletion induced by repeated high doses of (+)-Fen *in vivo* (Mennini *et al*., 1996).

Conclusions

Our data suggest that a common mechanism underlies (+)-Amph-, pCA- and MDMA-induced [^{3}H]-dopamine release, as well as (+)-Fen-, pCA- and MDMA-induced [^{3}H]-5-HT release, all of them being partially Ca^{2+}-dependent (exocytotic-like) and partially Ca^{2+}-independent presumably through the neurotransporter. We confirmed that the selectivity for 5-HT or dopamine release is very likely due to their selective interaction, as substrates, with the transporter proteins. With regard to Ca^{2+}-dependent release, a common target which is methiothepin-sensitive, may mediate the action of both 5-HT- and dopamine-releasing amphetamines. Characterizing this target will help us to better understand the pharmacological and toxicological effects of (+)-Amph and its derivatives, and of methiothepin.

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