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SIMULTANEOUS EVALUATION BY A DOUBLE LABELLING METHOD OF DRUG-INDUCED UPTAKE INHIBITION AND RELEASE OF DOPAMINE IN SYNAPTOSOMAL PREPARATION OF RAT STRIATUM

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Abstract—Crude synaptosomal preparations from corpora striata of rat were preloaded with [^{14}C]DA, rinsed, and then incubated with [^{3}H]DA and the drug to be tested. During the 5 min of the second incubation, DA uptake and release rates were time- and temperature-dependent. On this double labelling test, nomifensine, cocaine, benztropine and amphetamines displayed IC_{50} and release curves similar to those obtained from separate uptake and release studies. In the presence of high concentrations of iprindole, amitriptyline and butriptyline ($>3 \times 10^{-6}$ M), a [^{14}C]DA release was observed which closely coincided with an apparent inhibition of [^{3}H]DA uptake. This double labelling test allows the determination of the participation of the releasing effect of drugs in their apparent inhibition of DA uptake.

Some indirect dopamine (DA) agonists such as nomifensine, cocaine and benztropine behave as inhibitors of the DA reuptake without affecting, at least notably, the release of newly accumulated DA [1–4]. This is not the case for a drug such as amphetamine which simultaneously inhibits DA reuptake and induces DA release. This latter effect might lead to an erroneous evaluation of the effect of the drug on the reuptake; thus a releasing agent could therefore appear as an uptake inhibitor [5–6].

We have developed a double labelling test, using a synaptosomal preparation of rat striatum, in order to assess simultaneously the efflux and the uptake of DA and so determine the participation of the releasing effect elicited by some drugs in their apparent inhibition of DA uptake.

MATERIALS AND METHODS

Male Sprague–Dawley rats (Charles River, Saint Aubin les Elbeuf) weighing 150–200 g were decapitated in a cold room (4°). The brains were rapidly removed and the corpora striata dissected on a cold surface as described by Glowinski and Iversen [7]. The striata were homogenized in a glass Teflon homogenizer (Potter–Elvehjem type, clearance 0.08–0.13 mm) in 10 volumes (w/v) of ice-cold 0.32 M sucrose. Homogenizations were performed with 10 up-and-down strokes with a pestle revolution of 800 rpm. The pestle and glass vessel were rinsed with 10 volumes of ice-cold sucrose which were added to the homogenate. The nuclei and cell debris were separated by centrifugation (1000 *g*, 10 min, 4°). The supernatant was gently stirred to yield a uniform suspension which served as a source of the crude synaptosomal preparation.

Double labelling test. A 400 μl aliquot of synaptosomal preparation was added to a test tube containing 3600 μl of Krebs–Ringer phosphate buffer. The final composition of the incubation medium included: 109 mM NaCl, 3.55 mM KCl, 2.4 mM $CaCl_2$, 0.61 mM $MgSO_4$, 1.1 mM KH_2PO_4, 25 mM $NaHCO_3$, 5.4 mM glucose and 20 μM nialamide at pH 7.4. The incubation medium was bubbled with a 95%O_2–5%CO_2 gas mixture for 30 min before use to ensure complete oxygenation. Following a 5 min preincubation at 37° in a shaking water bath under an atmosphere of 95%O_2–5%CO_2, 125×10^{-8} M [^{14}C] DA was added (40 μl) and the incubation was continued for 5 min. The reaction was stopped by centrifugation (7000 *g*, 10 min, 4°), the pellet was washed by resuspension in 4 ml of incubation medium; after centrifugation the supernatant was discarded and the pellet was resuspended in 460 μl of ice-cold medium. Following a 5 min preincubation, 100 μl of ice-cold suspension of prelabelled preparation was added to 900 μl of the previously described incubation medium, already containing 10 μl of [^{3}H]DA (final concentration 10^{-8} M) with or without the drug to be tested. Incubation was continued at 37° in a shaking water bath under an atmosphere of 95%CO_2–5%CO_2 for 5 min. The reaction was stopped by centrifugation (7000 *g*, 10 min, 4°). A 800 μl portion of supernatant was added to 5 ml of Aqualuma® in order to determine the amount of released [^{14}C]DA. The final pellet was homogenized by sonication in 250 μl of distilled water: an aliquot of this homogenate was counted for determination of the radioactivity; the amount of protein in each incubation tube was estimated by the method of Lowry *et al.* [8].

The uptake and the release at 0° were also studied for each experiment in order to determine the temperature-sensitive uptake and release. The specific

uptake of DA was defined as the differences in fmole DA/mg protein after incubation at 37° (total uptake) and 0° (non-specific uptake). Uptakes at 37° and 0° were corrected for dilution of [^{3}H]DA by respectively [^{14}C]DA released at 37° and 0°; the calculations took into account the temperature-dependent time-course of the [^{14}C]DA release. For each batch of experiments, spontaneous and [^{3}H]-DA-induced release were determined. [^{14}C]DA ratios (R) of supernatant/supernatant + pellet were calculated as fmol/ml of medium to fmole in medium + fmole in pellet. The release percentage of DA was calculated according to the formula:

$$\frac{R_D - R_{3H}}{1 - R_{3H}} \times 100,$$

where R_D is the ratio evaluated from incubations with the tested drug and [^{3}H]DA, and R_{3H} the ratio evaluated from the control incubation with [^{3}H]DA alone. RC_{50} (defined as the drug concentration releasing 50% of the [^{14}C]DA which is present in the synaptosomal preparation at the end of its incubation in presence of [^{3}H]DA alone) was calculated by regression analysis (least-squares method) of DA release percentages. The uptake inhibition was corrected for dilution of [^{3}H]DA by released [^{14}C]DA and expressed as a percentage of controls; this correction was significant for drugs eliciting a DA release. IC_{50} (drug concentration inhibiting 50% of control uptake) were calculated by regression analysis. Mean ± S.E.M. were compared by Student's *t*-test.

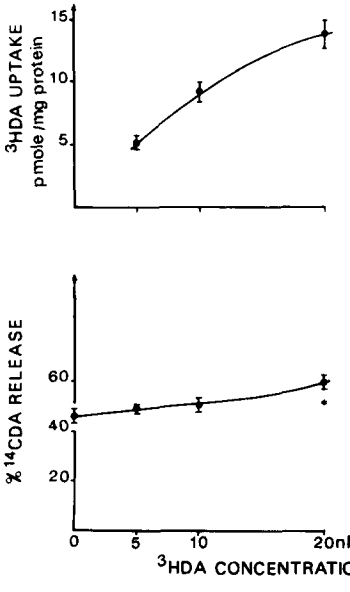

Fig. 1. Effect of [^{3}H]DA concentration on its synaptosomal uptake and the [^{14}C]DA release during the second incubation. Upper panel: specific uptake for various [^{3}H]DA concentrations; lower panel: spontaneous and [^{3}H]DA-induced release of [^{14}C]DA. Synaptosomal preparation was preloaded with [^{14}C]DA (1.25 μM), rinsed, and incubated at 37° in the presence of 0, 5, 10 or 20 nM [^{3}H]DA. Specific [^{3}H]DA uptake was obtained by difference of accumulations after incubations at 37° and 0°. Percentages of [^{14}C]DA release were calculated as [^{14}C]DA present in supernatant/[^{14}C]DA in supernatant + pellet. Each point is the mean ± S.E.M. of three determinations. (*) Significantly different from spontaneous release ($P < 0.05$).

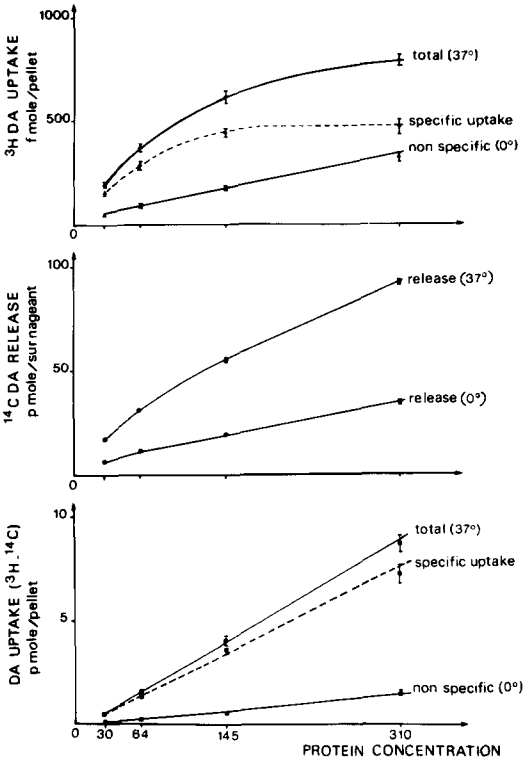

Fig. 2. Effect of the protein concentration on synaptosomal uptake and release of dopamine during the second incubation. Upper panel: total, specific and non-specific uptakes of [^{3}H]DA; middle panel: [^{14}C]DA release at 37° and 0°; lower panel: total, specific and non-specific uptakes of DA ([^{3}H]DA corrected for dilution by the released [^{14}C]DA). Synaptosomal preparation was preloaded with [^{14}C]DA (1.25 μM), rinsed and, after centrifugation, resuspended in 450 μl of ice-cold Krebs–Ringer medium. 100 μl of original or diluted suspension (respectively; 1/2, 1/4, 1/8) was added to 900 μl of incubation medium preincubated at 37° or 0° and already containing 10 nM [^{3}H]DA. The second incubation was continued at 37° or 0° for 5 min and stopped by centrifugation. Each point is the mean ± S.E.M. of eight determinations.

In some experiments, aliquots of ice-cold suspension of [^{14}C]DA prelabelled preparation were incubated at 37° for 2 min with [^{3}H]DA 10^{-8} M and immediately filtered on Millipore discs (DAWP type). The filters were washed with 5 ml of incubation medium at 37° and subsequently superfused for 5 min with medium at 37° at a rate of 0.5 ml/min with or without 3×10^{-5} M (+)amphetamine. Thus this superfusion started respectively 1 min and 29 min after the end of incubations with [^{3}H]DA and [^{14}C]DA.

Uptake studies. A 50 μl aliquot of crude synaptosomal preparation was preincubated as previously described. Incubation was continued with 2×10^{-8} M [^{3}H]DA for 5 min and the reaction was stopped by adding 2 ml of ice-cold medium to each incubation tube and then cooling the tube in ice. After centrifugation (7000 *g*, 10 min, 4°), the pellet

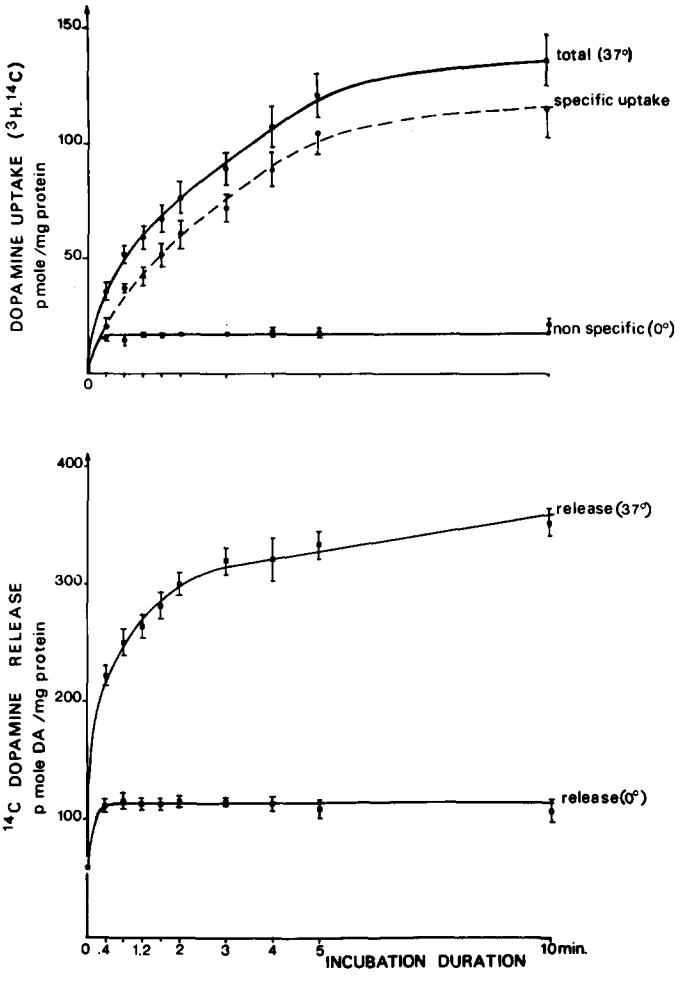

Fig. 3. Time course of release and uptake of dopamine by a [^{14}C]DA preloaded synaptosomal preparation during a second 10 min incubation. Lower panel: time course of the [^{14}C]DA release at 37° and 0°, upper panel: total, specific and non-specific uptakes of DA ([^{3}H]DA corrected for dilution by released [^{14}C]-DA). Synaptosomal preparation was preloaded with [^{14}C]DA (1.25 μM), rinsed, and incubated at 37° or 0° for various times (abscissa) with [^{3}H]DA 10 nM. DA uptake was corrected for dilution of [^{3}H]DA by released [^{14}C]DA. The DA released at time 0 was 61 ± 2 pmoles/mg. Each point is the mean ± S.E.M. of four determinations.

was washed and the suspension recentrifuged. The final pellet was treated as previously described.

Release studies. Procedures for preparation of the prelabelled synaptosomal suspension were the same as described in the double labelling study section, except that in the first incubation [^{3}H]DA 1.25×10^{-7} M was added instead of [^{14}C]DA. The second incubation occurred without the addition of DA. The release percentage of DA was calculated as described above except that R_C (ratio evaluated from control incubations) substituted for R_{3H}.

Drugs. [^{3}H]DA HCl (6 Ci/mmole) was purchased from Amersham France and [^{14}C]DA acetate (53 mCi/mmole) from C.E.N. Saclay. Cocaine HCl was purchased from la Cooperative Pharmaceutique Française. The following drugs were generously donated by manufacturers: (+) and (−)amphetamine sulfate (Smith, Kline & French); pOH amphetamine HBr (Cassenne); nialamide (Pfizer); nomifensine maleate (Hoechst); amitriptyline HCl and benztropine mesylate (Merck, Sharp & Dohme–Chibret); butriptyline HCl (Auclair); iprindole HCl (Wyeth–Byla).

RESULTS

Accumulation and release of dopamine during the second incubation. Effect of [^{3}H]*DA concentrations on uptake and release of DA in the double labelling test*

The increase in [^{3}H]DA concentration (5–20 nM) did not lead to a proportional increase in the specific [^{3}H]DA uptake; it elicited a low [^{14}C]DA release. This [^{14}C]DA release became significant only for a [^{3}H]DA concentration higher than 10 nM (Fig. 1).

Effect of protein concentration on the uptake and release of DA during the second incubation

When different concentrations of synaptosomes

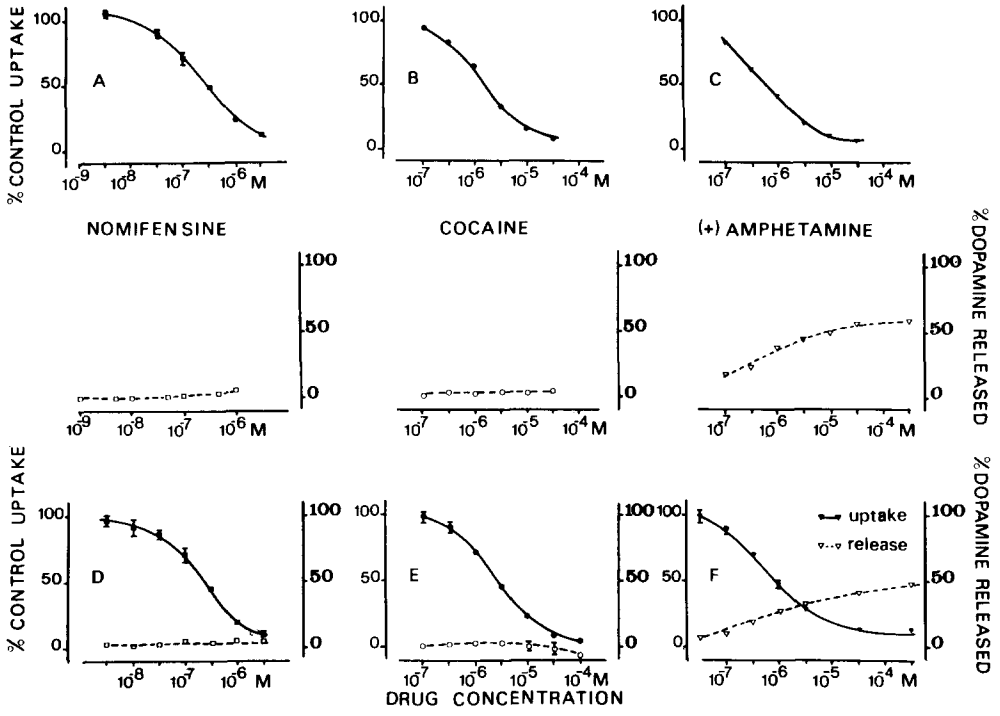

Fig. 4. Comparison between the effect of nomifensine, cocaine and (+)amphetamine on the uptake and release of DA determined either separately or by the double labelling test. Upper panels: separate determinations of [^{3}H]DA uptake inhibition; middle panels: separate determinations of drug-induced release of [^{3}H]DA; lower panels: simultaneous determinations of [^{3}H]DA uptake and [^{14}C]DA release by double labelling test. The three sets of experiments were performed on the crude synaptosomal preparation obtained from striatum. For [^{3}H]DA uptake studies, the synaptosomal preparation was incubated at 37° for 5 min with [^{3}H]DA (20 nM) in the presence of increasing concentrations of drugs; for [^{3}H]DA release studies, the synaptosomal preparation was preloaded with [^{3}H]DA (125 nM), rinsed and therefore incubated at 37° for 5 min in the presence of increasing concentrations of drugs; for double labelling test, the synaptosomal preparation was preloaded with [^{14}C]DA, rinsed and then incubated at 37° for 5 min with [^{3}H]DA in the presence of increasing concentrations of drugs. Each point is the mean ± S.E.M. of 3–6 determinations. The 100% values of [^{3}H]DA uptake were (in pmoles/mg protein) for A: 27.3 ± 2.0, B: 24.6 ± 1.6, C: 29.8 ± 2.2, D: 6 ± 0.3, E: 7.4 ± 0.7, F: 8.9 ± 0.3.

preloaded with [^{14}C]DA were incubated with [^{3}H]-DA 10 nM during a 5 min period we noted that (i) the specific [^{3}H]DA uptake was not linear as a function of protein concentration (Fig. 2, upper panel), (ii) the [^{14}C]DA released at 37° increased more rapidly with protein concentration than the [^{14}C]DA released at 0°, and (iii) the specific DA uptake (specific [^{3}H]DA uptake corrected for dilution by released [^{14}C]DA) was linear as a function of protein concentration (Fig. 2, lower panel).

Time-course of the release and uptake of DA during a 10 min second incubation

Twenty seconds after the beginning of the second incubation, the [^{14}C]DA release occurring at 0° was at its maximal level which corresponded to about 20% of the total [^{14}C]DA present in the preloaded preparation. The release occurring at 37° is clearly higher since, after a 5 min incubation, it reached about 55% of the total [^{14}C] stored (Fig. 3, lower panel). Between 2 and 10 min of incubation, the [^{14}C]DA release at 37° was linear as a function of time ($R = 0.942$). The DA 'accumulation' occurring at 0° was low and was at its maximal level in about 20 sec. The DA uptake measured at 37° was much higher; it increased with time and was apparently saturated after 5 min of incubation (Fig. 3, upper panel). From the second minute up to the higher incubation time considered (10 min), the [^{14}C]DA release at 37° (54.7 ± 2.6 pmoles/mg) equalled the specific DA uptake (54.6 ± 4.5 pmoles/mg).

Evaluation of drug effect on uptake and release of DA determined either separately or in the double labelling test

Nomifensine, cocaine (Fig. 4) and benztropine inhibited the DA uptake at the same respective concentrations either in separate uptake studies or in the double labelling tests; thus in the separate uptake studies and in the double labelling test the IC_{50} (μM) were respectively: 0.29 ± 0.02 and 0.22 ± 0.03 for nomifensine, 1.62 ± 0.13 and 1.41 ± 0.10 for cocaine and 0.24 ± 0.02 and 0.24 ± 0.04 for benztropine. In the double labelling test the IC_{50} of (+)amphetamine (0.98 ± 0.08 μM) was somewhat higher than in the separate uptake study (0.70 ± 0.07 μM). Both in release studies and double labelling tests, neither nomifensine

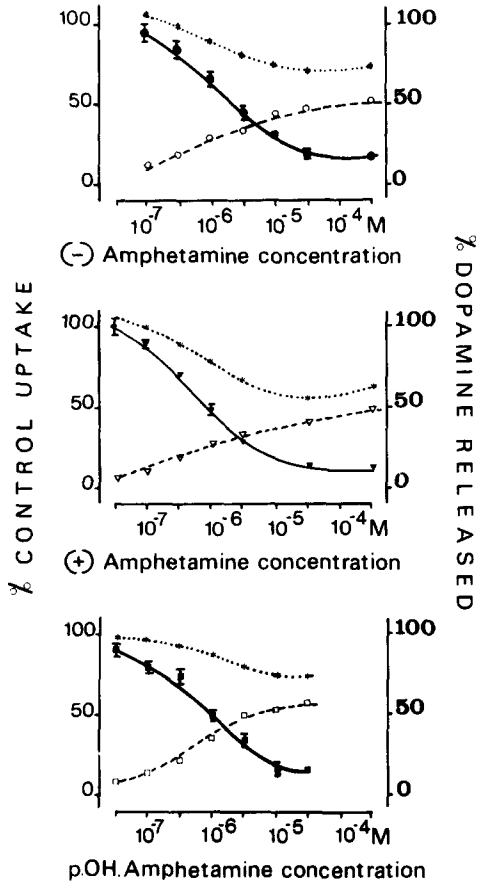

Fig. 5. Effects of (+) or (−) amphetamine and pOH amphetamine on the uptake and release of DA determined by the double labelling test. Synaptosomal preparation was preloaded with [^{14}C]DA (1.25 μM), rinsed and incubated at 37° for 5 min with [^{3}H]DA (10 nM) in the presence of increasing concentrations of drugs. Open symbols, DA release; filled symbols, DA uptake corrected for dilution of [^{3}H]DA by released [^{14}C]DA; (*) inhibited DA uptake minus DA release. Each point is the mean ± S.E.M. of three determinations.

3×10^{-6} M nor cocaine 10^{-4} M increased the [^{14}C]-DA concentration in the incubation medium. Benztropine exhibited a releasing effect at concentrations higher than 3×10^{-7} M; this effect was moderate and reached 18% of the releasable [^{14}C]DA at 3×10^{-6} M when the uptake inhibition was almost complete. The releasing effect of (+)amphetamine appeared very similar with both methods of determination (Fig. 4).

Effects of (+), (−) and pOH amphetamine on DA release and uptake assessed by the double labelling test

The IC_{50} for inhibition of the [^{3}H]DA uptake was respectively 0.98 ± 0.08 μM for (+)amphetamine, 2.91 ± 0.65 for (−)amphetamine and 1.03 ± 0.20 for pOH amphetamine (Fig. 5). In the absence of a 100% release of [^{14}C]DA, in order to compare the relative efficacy of these agents, we determined the percentages of DA released by drugs at 3×10^{-5} M: they were 42 ± 1.5% for (+)amphetamine, 46 ± 1% for (−)amphetamine and 58 ± 1% for pOH amphetamine ($P < 0.01$ as compared to (+)amphetamine). To check whether the DA previously accumulated was released similarly to the DA more recently uptaken, the following experiment was carried out. A synaptosomal preparation, successively labelled with [^{14}C]DA (29 min before) and [^{3}H]DA (1 min before), was superfused with (+)amphetamine 3×10^{-5} M. The release of the two labelled DA was 21 ± 6% for [^{14}C]DA and 23 ± 5% for [^{3}H]DA (percentages of DA stored at the beginning of superfusion). Since a similar release of the two labelled DA was observed, we have indicated in Fig. 5 the curve resulting from subtraction of the percentage of released DA from that of the apparent inhibition of DA uptake. However, for a drug concentration producing an almost complete blockade of uptake, the releasing effect was only about 50% of the accumulated [^{14}C]DA.

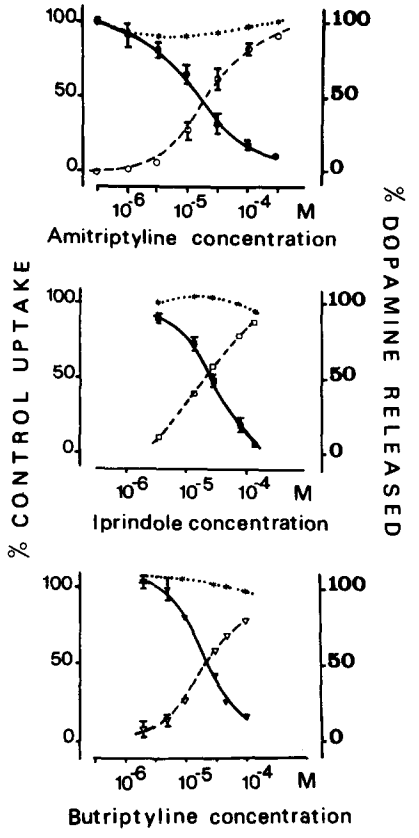

Fig. 6. Effects of amitriptyline, butriptyline and iprindole on the uptake and release of DA determined by the double labelling test. Synaptosomal preparation was preloaded with [^{14}C]DA (1.25 μM), rinsed and then incubated at 37° for 5 min with [^{3}H]DA (10 nM) in presence of increasing concentrations of drugs; open symbols, DA release; filled symbols, DA uptake corrected for dilution of [^{3}H]DA by released [^{14}C]DA; (*) inhibited DA uptake minus DA release. Each point is the mean ± S.E.M. of three determinations.

Effects of various antidepressant agents on the release and uptake of DA determined by the double labelling test

Amitriptyline, butriptyline and iprindole induced an inhibition of [^{3}H]DA uptake which coincided with a release of [^{14}C]DA. This release affected about 90% of the accumulated [^{14}C]DA for drug concentrations which almost completely blocked the [^{3}H]DA uptake. For these drugs, IC_{50} and RC_{50} were two orders of magnitude higher than those determined for nomifensine or benztropine; they were respectively 17.5 ± 4.6 μM and 20.1 ± 3.8 μM for amitriptyline, 25.5 ± 1.6 and 19.3 ± 4.8 for butriptyline and 22.9 ± 1.9 and 18.1 ± 1.2 for iprindole (Fig. 6).

DISCUSSION

The double labelling test assesses in one step both the DA uptake and the release of the newly-accumulated DA; it allows rapid determination of whether a drug acts as an uptake blocker and/or a releaser of DA.

In this test the time course of the release during the second incubation developed in two phases: the first one, corresponding to the first 2 min, was characterized by an important [^{14}C]DA release (about 50% of the [^{14}C]DA present in the preloaded preparation) and the second one, between 2 and 10 min, by a very low rate of efflux. During this second phase the [^{14}C]DA release rate was linear as a function of time. After 20 sec of the second incubation, the [^{14}C]DA release proceeded only of a thermodependent mechanism. The first phase most likely corresponds to the recovery of the DA concentration gradient between the intrasynaptosomal medium and the incubation buffer; nevertheless, considering the water volume of the synaptosomal preparation (inferred from the amount of protein [9–10]), one may calculate that after a 5 min incubation with [^{3}H]DA 10 nM, the [^{14}C]DA is 400–500 fold more concentrated in the synaptosomal fraction than in the medium. The time course of the DA uptake during the second incubation indicates an obvious accumulation during the first 5 min with an apparent saturation for a higher period.

From these data, we have selected a 5 min time for the second incubation; thus the initial release of [^{14}C]DA vanished and the maximal accumulation capacity of DA by the synaptosomal preparation was reached. Increasing concentrations of [^{3}H]DA during the second incubation led to a non-proportional accumulation of [^{3}H]DA. For a higher tested [^{3}H]DA concentration (20 nM), the [^{3}H]DA-induced release of [^{14}C]DA was significant. Therefore the [^{3}H]DA concentration we used ensured a sufficient [^{3}H]radioactivity in the pellet but was insufficient to produce a significant [^{14}C]DA release. The synaptosomal density (or protein concentration) was not critical since the DA uptake was proportional to the protein concentration at the studied concentrations. The protein concentration of the sample was 150–200 μg/pellet.

For all the tested compounds, whether their DA releasing effect and their uptake inhibiting potency (IC_{50}) were determined simultaneously (by double labelling test) or separately, the individual values obtained were almost similar and in agreement with values already reported [11–14]. Nomifensine and benztropine are indeed the most potent inhibitors of the DA uptake which were tested, while cocaine is less active. (+)-Amphetamine appears less effective in the double labelling test than in the uptake study; this difference is due to the correction for dilution of [^{3}H]DA by the released [^{14}C]DA. This emphasizes that the uptake studies lead to an overestimation of the inhibiting potency of the releasing drugs since the amount of released endogenous amine is unknown. Neither nomifensine nor cocaine cause a significant increase in [^{14}C]DA in the medium, even at drug concentrations which completely block the DA uptake; however, an incubation time of 10 min leads to a significant increase in [^{14}C]DA in the incubation medium (unpublished data). As suggested by some authors [1–3], benztropine from 3×10^{-7} M induces a concentration-dependent DA release which becomes significant when the DA uptake is almost completely blocked. Compared to nomifensine and cocaine, it appears that benztropine induces a true DA releasing effect at the higher tested concentration (10^{-5} M); nevertheless the blockade of DA accumulation is clearly the most important component of benztropine's effect.

The [^{14}C]DA releasing effect of (+) amphetamine in the double labelling test is similar to that reported by Fischer and Cho [15] who operated on the classical single labelling test. It was saturated at about 50% of the stored DA. In superfusion, the amphetamine releasing effect was lower (about 22%) in agreement with values obtained by Raiteri *et al.* [16]. Thus the differences in experimental models seem to account for these discrepancies; this is strengthened by experiments reported by Heikkila *et al.* [17] operating on slices and indicating a very important releasing effect at the end of a 15 min incubation period (about 90%). Most authors consider that the releasing effect of (+)amphetamine is limited to a cytosolic pool of DA: this fact might account for this partial release [18–19]. When we subtract the percentage of the DA released by the amphetamine-like drugs from that of the apparent inhibition of DA uptake, the blockade of accumulation appears relatively weak. This suggests that the releasing effect of these drugs could be an important component of their indirect dopaminergic agonist property. The subtraction operated presumes that the recently uptaken [^{3}H]DA (1 min) may be released by drugs in the same proportion as the [^{14}C]DA stored 29 min previously. Such a point of view is supported by our superfusion studies. Nevertheless, since this experiment did not reproduce exactly the conditions of the double labelling test, it cannot be excluded that the extemporaneously uptaken DA might participate to a different releasable pool than that corresponding to the DA stored a few minutes before, as suggested by studies with a high rate of superfusion [20]. Several antidepressant drugs have been claimed as inhibitors of the DA uptake in studies which considered only the blockade (for relatively high concentrations) of the DA accumulation into striatal synaptosomes [12, 21]. On the double labelling test, there is clear simultaneity between the apparent inhibition of [^{3}H]DA uptake

and the release of [^{3}H]DA. These antidepressants produced an almost complete release of the stored DA. The comparison with the releasing effect of amphetamine could suggest that the DA release elicited by antidepressants concerns various pools of synaptosomal DA. There is also a clear similarity between their respective IC_{50} and RC_{50} values; the subtraction of the percentage of the released DA from that of the apparent inhibition of DA uptake establishes that these drugs have no inhibitory effect on the DA uptake and suggests that amitriptyline, butriptyline and iprindole are only releasing agents; we have extended this study in a twin paper.

In conclusion, the double labelling test allows a simultaneous determination of the uptake inhibition and release of DA on the same biological material. It provides IC_{50} and RC_{50} values similar to those measured in separate uptake and release studies as long as the drug is devoid of releasing effect. This test clearly demonstrates the possible interference between the uptake inhibition and the release of newly accumulated DA. Thus it allows more precise determination of the true uptake inhibition by suppressing the interference due to the releasing effect of drugs.

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