

The in vivo modulation of dopamine synthesis by calcium ions: influences on the calcium independent release

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Abstract

To investigate the contribution of the dopamine (DA) synthesis to both the *calcium-dependent* and the *carrier-mediated*, mechanisms of DA release in the striatum, anaesthetized rats were locally superfused in the striatum with a push–pull cannula supplied with an artificial CSF containing tritiated tyrosine. DA, dihydroxyphenylacetic acid (DOPAC) and their respective specific activity were measured in effluent and used to evaluate changes in the DA synthesizing rate. Excluding calcium ions from the CSF only partially reduced spontaneous DA release (70%) still leaving a possible *carrier-mediated* DA release. This effect was not additive with a local superfusion with 0.1 mM *α*-methyl-*p*-tyrosine, a blocker of DA synthesis, suggesting that synthesis could already be reduced by calcium-free superfusion. Local superfusion with 100 μM cadmium in the presence or not of calcium ions, increased the DA release (220 and 350%, respectively), simultaneously reducing DA synthesis. Local application of 1 μM calcium ionophore (A23187) was without effect on the basal release of DA but enhanced DA synthesis and increased the amphetamine-evoked and carrier-mediated amine release. We conclude that DA synthesis can be a modulatory process of the firing-independent and *carrier-mediated* amine release while it weakly affects the classical *calcium-dependent* release. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Elucidating the mechanisms which control neurotransmitter metabolism and release is a crucial step toward the understanding of nerve terminal function. It has already been shown that the intracellular level of calcium ions (Ca^{2+}) has an important influence on neurotransmitter release (Augustine et al., 1987; Katz and Miledi, 1970; Mulkey and Zucker, 1991; Smith and Augustine, 1988) and on enzyme activities (El Mestikawy et al., 1983; Haycock et al., 1984; Kapatoss and Zigmond, 1982). Since transient Ca^{2+} increases within nerve terminals are spatially restricted (Lipscombe et al., 1988; Miller, 1991) it may be hy-

pothesized that Ca^{2+} separately affects multiple processes within the different ultrastructural compartments of the nerve terminal (Meldolesi et al., 1988). Dopamine (DA) metabolism and release in the striatum constitute adequate experimental models to investigate potentially distinct effects of Ca^{2+} . In dopaminergic neurons, DA synthesis depends on the activity of tyrosine hydroxylase (TH, EC 1.14.16.2), the rate-limiting enzyme in its synthetic pathway (Levitt et al., 1965). This enzyme is activated by phosphorylation under the control of various protein kinases including the Ca^{2+} /phospholipid-dependent protein kinase PKC (Albert et al., 1984; Pasqualini et al., 1994) and the Ca^{2+} /calmodulin-dependent protein kinase PKII (El Mestikawy et al., 1983; Haycock, 1993; Zigmond et al., 1989). Furthermore, the release of DA from terminals involves two different mechanisms (for a review see Grace, 1991). The first, exocyto-

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sis, is triggered by the firing of DA neurons, and has been demonstrated to be closely dependent of Ca^{2+} ions (Leslie et al., 1985; Westerink et al., 1988). It will be presently referred as the Ca^{2+} -dependent releasing process. The second is not directly dependent of Ca^{2+} and involves the reversal of the DA reuptake mechanism since it can be blocked by the presence of reuptake blockers such as GBR 12909 (Olivier et al., 1995) or nomifensine (Fischer and Cho, 1979; Raiteri et al., 1979; Soares-Da-Silva and Garrett, 1990a; Woodward et al., 1988). It will be referred here as the *carrier-mediated* releasing process. Recently, these two releasing processes were directly and simultaneously observed *in vivo*. The Ca^{2+} -dependent DA release can be observed using voltammetry and is characterized by a very fast DA over-flow and clearance in response to electrical stimulations of DA axons. It involves however very low amine amounts since stimulating conditions in the physiological range are unable to modify durably the extracellular amine concentration. In opposite the *carrier-mediated* DA release produces long term alterations in the extracellular space, thus involving considerable masses of the amine (Olivier et al., 1995).

This discrepancy led us to question the role of the synthesis in the two releasing processes. Indeed intraterminal stores could supply for the Ca^{2+} -dependent amine release when the neosynthesis should be required for the *carrier-mediated* one. Thus, in this study, we have further investigated the control exerted by Ca^{2+} on both DA synthesis and on the two DA-releasing processes. Unfortunately, no technique, to date, allow to directly measure each of the two releasing processes. The global DA release was thus approached by using a push-pull cannula implanted in the striatum of anaesthetized rats to evaluate variations of the extracellular DA. The DA synthesis was indirectly observed. A local and continuous superfusion of the tissue with tritiated DA precursor, [^3H]tyrosine ([^3H]TYR), allowed to measure the release of [^3H]DA and the tritiated dihydroxy-phenyl-acetic acid ([^3H]DOPAC) neosynthesized. Indeed the dynamic alterations of these parameters are well correlated with the rate of DA synthesis (Leviel et al., 1989; Zetterström et al., 1988). Two types of treatments were devoted to discriminate the various processes influenced by Ca^{2+} in DA terminals. First, the effects of a reduction of Ca^{2+} entry into DA terminals was examined by Ca^{2+} removal in the superfusing fluid and the superfusion with cadmium (Cd^{2+}) known to block the Ca^{2+} -dependent release when favouring the *carrier-mediated* one. Furthermore, the effect of local application of *α*-methyl-*p*-tyrosine (αmpt), was compared to a Ca^{2+} free superfusion. Second, a forced entry was realized by a local application of the Ca^{2+} ionophore A23187. The consequences of this treatment were ana-

lysed by releasing massively the cytoplasmic amine with a local application of a high dose amphetamine before evaluating the previously mentioned parameters.

2. Materials and methods

2.1. Animals

Rats (Wistar male, Iffa-Credo, France) weighing 300 g were used in this study. They had free access to food and water and were housed on a 12/12 h light/dark cycle under constant temperature (21°C) and humidity (40%). Animals were maintained and killed in accordance with the European Communities Council Directive (86/609/EEC).

2.2. Animal preparation and *in vivo* superfusion

The animals were anaesthetized with halothane (1.5%) in pure oxygen and tracheotomized. They were implanted with a push-pull cannula in the anterior part of the caudate nucleus (Ant.: 8 mm; Lat.: 2.75 mm; Vent.: 6 mm), the horizontal zero reference plane passing through the interaural axis and incisor bar (Albe-Fessard et al., 1971). Cannulae (1.0 mm outer diameter) were supplied (flow rate: 12.5 $\mu\text{l}/\text{min}$) with an artificial CSF adjusted to pH 7.4 with an O_2/CO_2 (95:5 v/v) mixture. [^3H]TYR (3,5-[^3H]tyrosine, 50 Ci/mmol, Dositek, France) was purified by high performance liquid chromatography (HPLC) on a C18 Microbondapak column (Millipore-Waters, France) using H_3PO_4 (1 mM, pH 3) as a mobile phase. One hour after the beginning of the superfusion [^3H]TYR was added to artificial CSF (80 $\mu\text{Ci}/\text{ml}$) and superfusates collected thereafter as successive 20 min fractions. Substances were applied locally in the striatum by addition to the artificial CSF at the following concentrations: A23187 (Tocris Cookson) 1 μM ; αmpt (Sigma) 10 μM ; CdCl_2 (Sigma) 100 μM (to respect the osmolarity, Mg^{2+} concentration was balanced when Ca^{2+} was removed).

At the end of each experiment, to verify the cannula location, the animals were intracardially perfused with a 4% formaldehyde solution, the brain was removed, sliced (100 μm) and stained with cresyl violet.

2.3. Biochemical analysis

Endogenous and labelled DA and DOPAC were measured in the superfusing fluid. In brief, the DA and DOPAC concentrations in push-pull effluent were determined by electrochemical detection with potential of the working electrode at 0.8 V (Millipore-Waters 460) after HPLC separation (column C18 Brownlee

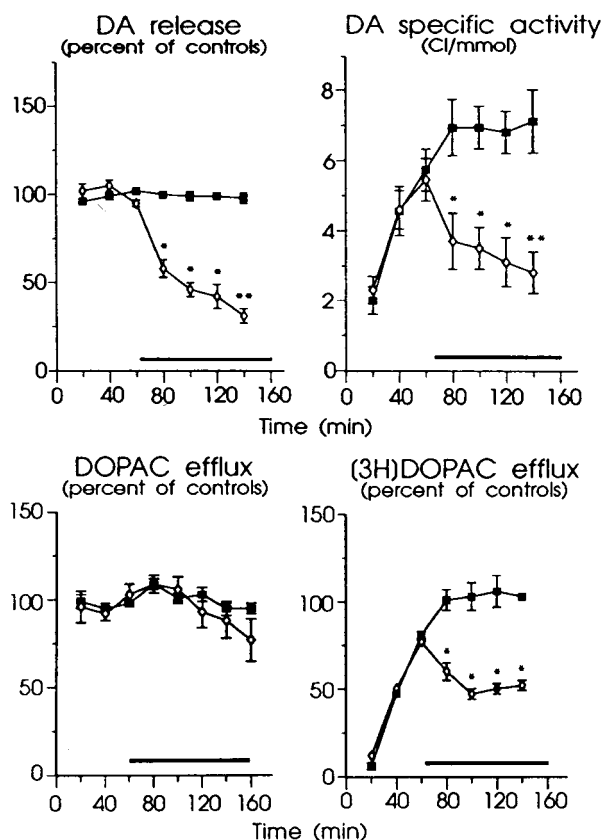


Fig. 1. Effects of the Ca²⁺ removal from the superfusing fluid on the release of DA, the DA_{sa} and efflux of DOPAC and [³H]DOPAC. Push-pull cannulae were implanted in the striata of anesthetized rats and supplied with artificial CSF containing L3,5[³H]tyrosine ([³H]TYR, 50 Ci/mmol, 80 μCi/ml, 12.5 μl/min). DA and DOPAC were measured in 20 min fractions (HPLC and electrochemical detection) and the radioactivity corresponding to the peaks was counted, allowing to calculate the DA_{sa} expressed in Ci/mmol. The Ca²⁺-free medium (black bar) was superfused 60 min after the beginning of the superfusion with [³H]TYR. Data are the means (±SEM) of six treated animals (◇) compared to nine controls (■). **p* < 0.05; ***p* < 0.01.

RP18, 5 μm, 2.1 × 220 mm, maintained at 25°C; Mobile phase: 100 mM NaH₂PO₄, 0.1 mM EDTA-Na₂, 0.28 mM sodium octyl sulfate, 6% methanol, adjusted to pH 3.3; flow rate 0.25 ml/min). The radioactivity corresponding to each HPLC peak was counted using a continuous flow scintillation detector (Packard-Radiomatic) to calculate DA specific activity (DA_{sa} in Ci/mmol). The yield of the superfusion procedure was measured in separated experiments by counting the radioactivity-ratio between blood and superfusates, 1 h after an intravenous injection of tritiated water (Elghozi et al., 1981) and was found to be 8%.

2.4. Data analysis

For an easier comparison and to limit the inter-indi-

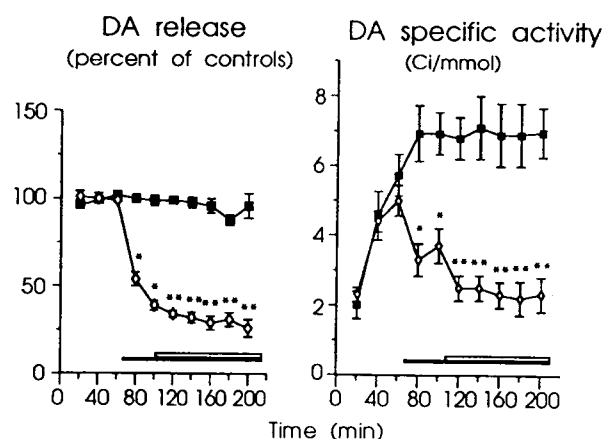


Fig. 2. Effects of α -methyl-*p*-tyrosine (α mpt) addition (open bar) to the Ca²⁺-free superfusion medium (black bar) on the DA release and DA_{sa}. As described in Fig. 1, Ca²⁺-free medium was superfused 60 min after the beginning of the superfusion with [³H]TYR; α mpt (100 μM) was added 100 min after the beginning of the superfusion and until the end of the experiment (200 min). Six treated animals (◇) were compared to nine controls (■). **p* < 0.05; ***p* < 0.01.

vidual fluctuations, the results concerning DA, DOPAC and [³H]DOPAC release are presented after standardization to 100% of the spontaneous release defined from the first three fractions (1 h). Statistical analysis was conducted using a two tailed Student's *t*-test by comparing the mean of corresponding fractions of control and treated groups. More details about techniques and data analysis are given elsewhere (Leviel et al., 1989).

3. Results

The control values for DA in extracellular space was 15 ± 0.7 nM (mean ± SEM) and a specific activity of 7.1 Ci/mmol after three hours of superfusion with [³H]TYR. For the DOPAC, the control values were respectively 990 ± 30 nM and 1.2 Ci/mmol.

3.1. Superfusion with calcium free medium and α mpt

Removing Ca²⁺ from the artificial CSF produced a sharp decrease in the extracellular DA collected every 20 min, down to about 30% of the basal values after 80 min. This effect was accompanied by a lowering of [³H]DA release and [³H]DOPAC efflux (Fig. 1). The effects of an α mpt application on these parameters have been detailed elsewhere (Leviel et al., 1989). The addition of 0.1 mM α mpt, 40 min after Ca²⁺ removal, did not further modify the effect of the superfusion in absence of Ca²⁺. The levels of DA reached about 30% of the spontaneous release after 80 min of treatment (Fig. 2). These two treatments did not produced

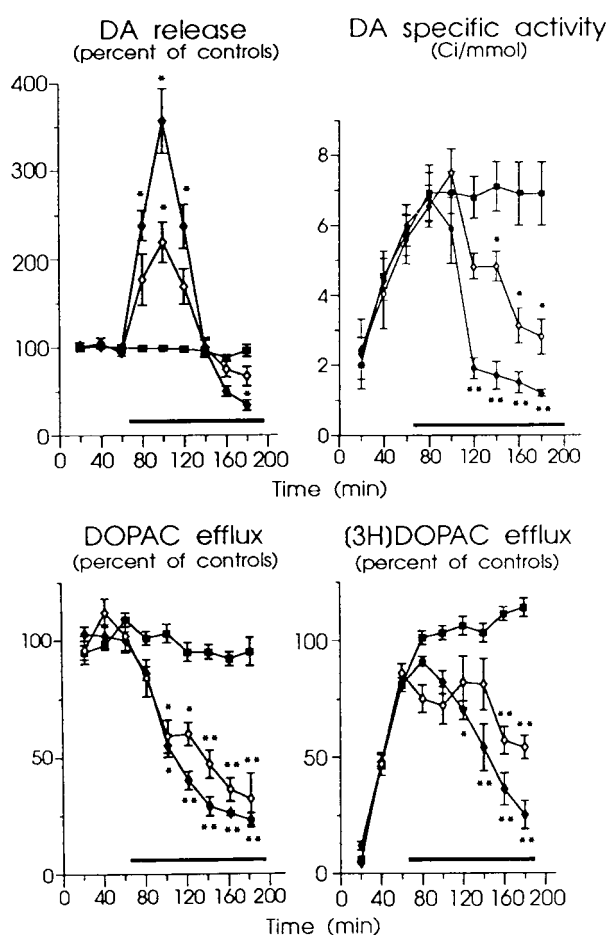


Fig. 3. Effects of the continuous application of Cd²⁺ in normal (◇) or Ca²⁺-free (◆) artificial CSF, on DA_{sa} and the release of DA, DOPAC and [³H]DOPAC. CdCl₂ (100 μM) was applied (black bar) 60 minutes after the beginning of the superfusion with [³H]TYR. Six treated animals in each group were compared to nine controls (■). **p* < 0.05; ***p* < 0.01.

additive effects and the lowering in DA_{sa} produced by the Ca²⁺-free superfusion was of the same amplitude than obtained previously with αmpt alone.

3.2. Blockade of calcium channels with Cd²⁺

The continuous application of 100 μM CdCl₂ in the Ca²⁺-free superfusing fluid produced a biphasic effect: a rapid increase in the spontaneous release of DA, up to 350% of the control values (Fig. 3), followed by a decrease in the DA release down to 50% of the control values after 120 min of treatment. Simultaneously a sharp decrease in [³H]DA occurred, lowering DA_{sa} values from 7 to 1 Ci/mmol while control values remained stabilized to 7 Ci/mmol. At the same time, the efflux of DOPAC and [³H]DOPAC were both reduced to 25% of the control values.

The effect of Cd²⁺ was also tested in the presence

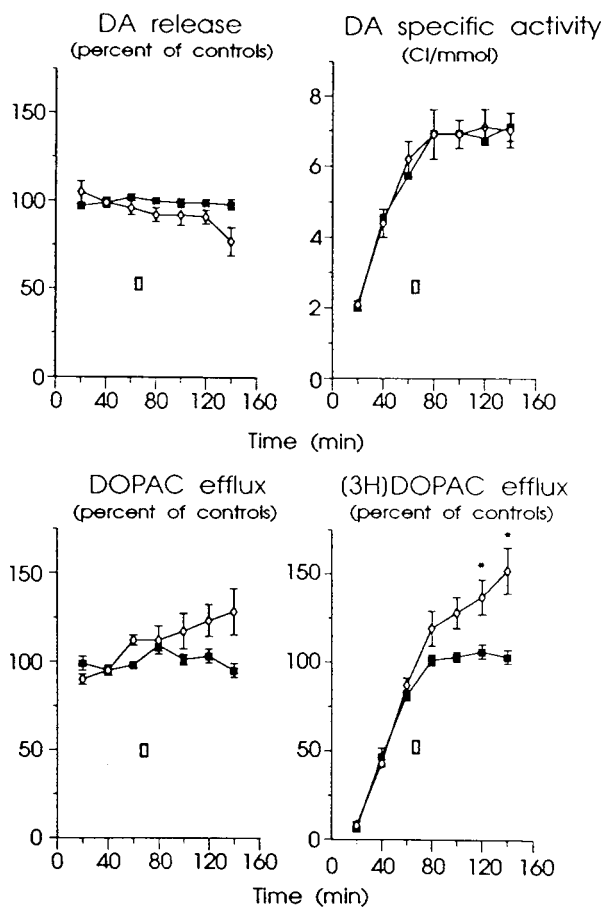


Fig. 4. Effects of the application (open rectangle) of Ca²⁺ ionophore A23187 (1 μM) on the release of DA, DOPAC, [³H]DOPAC and the DA_{sa}. A23187 was added during 20 min, 60 min after the beginning of the superfusion with [³H]TYR. Six treated animals (◇) were compared to nine controls (■). **p* < 0.05; ***p* < 0.01.

of normal (1.1 mM) Ca²⁺ (Fig. 3). As already reported (Olivier et al., 1995), the release of DA was also increased but to a lesser extent (220%), and the decrease in DA_{sa} was also less pronounced (3 Ci/mmol).

3.3. Superfusion with Ca²⁺ ionophore (A23187)

The Ca²⁺ ionophore A23187 (1 μM) added in superfusing fluid during 20 min, modified neither the spontaneous release of DA nor its specific activity (Fig. 4). However a moderate increase in DOPAC efflux (25%) together with a 50% increase in [³H]DOPAC efflux were observed.

To pin point on the effect of A23187 on the DA synthesis, the relative amounts of DA and [³H]DA were evaluated in the intraterminal cytoplasmic compartment 1 h after the treatment. A high dose amphetamine (1 mM) was thus used to massively exhaust this compartment allowing thus to examine its content. In

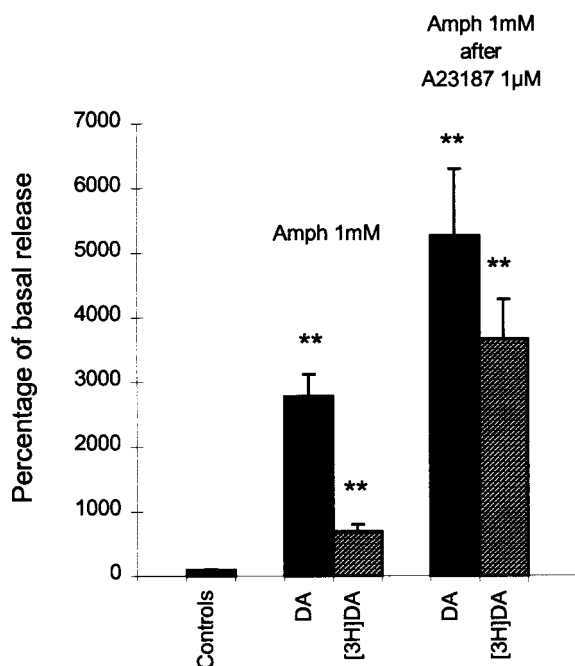


Fig. 5. Effects of a local 20 min application of amphetamine (1 mM) alone or 1 h after a local 20 min pretreatment with 1 μ M calcium ionophore A23187. These effects were observed on the release of DA and [3 H]DA during the 20 min of the amphetamine application. Six treated animals were compared to nine controls. * $p < 0.05$; ** $p < 0.01$ when compared to basal levels.

Fig. 5 are presented the effects, on the extracellular DA and [3 H]DA, of a 1 mM amphetamine application, alone or 1 h after, a pretreatment with 1 μ M Ca^{2+} ionophore A23187. When amphetamine was applied alone, during the 20 min treatment DA and [3 H]DA were respectively increased in extracellular space by $3422 \pm 302\%$ and $561 \pm 52\%$ when compared to basal values. One hour after 1 μ M A23187 treatment DA and [3 H]DA were respectively increased by $5272 \pm 826\%$ (1.54 fold the effect of amphetamine alone) and $3719 \pm 569\%$ (6.63 fold the effect of amphetamine alone) when compared to basal values. These observations evidenced that a pretreatment with A23187 enriched intraterminal DA in its neosynthesized form i.e. the [3 H]DA.

4. Discussion

Present results show that DA synthesis is not equally involved in the two DA releasing mechanisms. Paradoxically, the *carrier-mediated* mode of release appears to be the most closely related with DA synthesis in the terminals, being consequently, strongly, even indirectly, regulated by Ca^{2+} .

The push-pull superfusion technique was chosen because this method is based on a liquid/liquid

exchange at the tip of the cannula which allows a stable and reproducible labelling of the tissues with the tritiated precursors (that can not be obtained with dialysis probe) and produces a very restricted lesioned area (about 1 mm 3).

In our experiments, the enzymatic activity of TH was not directly measured but alterations in synthesis were revealed by the dynamic variations of [3 H]DA and [3 H]DOPAC efflux. Indeed the specific activity of DA can be considered as an index of the DA synthesis (Herdon et al., 1985) since the newly synthesized DA has a higher specific activity than the stored amine (Leviel et al., 1989). The total amount of DOPAC in extracellular space being in the range of the μ molar range cannot be directly related to the DA release (in the nmolar range). This could be due to the various origin of this substance. However, [3 H]DOPAC efflux can be considered as a good index of the amine synthesis, since, according to many authors, extracellular DOPAC comes from the metabolism of an unreleased and recently synthesized pool of dopamine (Herdon et al., 1985; Leviel et al., 1989; Soares-Da-Silva, 1987; Soares-Da-Silva and Garrett, 1990b; Zetterström et al., 1988).

The removal of Ca^{2+} from the superfusing fluid induced a partial decrease in the extracellular DA. This effect likely resulted from a partial reduction of the Ca^{2+} -dependent mechanism of release, the remaining DA efflux being thus, also partly, imputable to the *carrier-mediated* one (Ca^{2+} -independent). That does not mean that in normal conditions the ratio between the two mechanisms of release be of 70%/30%, their functional linkage is largely unknown and likely dependent of the environmental conditions. Indeed Cd^{2+} application was shown to induce an increase of the *carrier-mediated* release (Olivier et al., 1995) when tetrodotoxine application in the region of the medial forebrain bundle did not (Keefe et al., 1992). Nevertheless these two experimental conditions lead to a complete blockade of the Ca^{2+} -dependent release.

4.1. The Ca^{2+} -dependent DA synthesis

It can be argued from the present observations that in absence of Ca^{2+} in the superfusing fluid, the DA synthesis was largely impaired. First, it was demonstrated from a long time (Glowinski, 1973) that the DA, spontaneously released, is originating from an intraterminal compartment constituted by newly synthesized molecules (in our case with a high specific activity). Presently, the intraterminal compartment originating the released DA was likely different since [3 H]DA release and [3 H]DOPAC efflux were reduced evidencing the involvement of the intraterminally stored amine with a low specific activity. Thus, even to maintain a low rate of DA release, the stored amine

had to be involved suggesting a reduced synthesis. Second, the addition of a TH inhibitor, did not further decrease the extracellular DA or DA_{sa} also suggesting that the activity of TH is already inhibited by Ca²⁺ removal. Finally, blocking Ca²⁺ channels with Cd²⁺ reduced DA_{sa}, DOPAC and [³H]DOPAC and a delayed reduction in DA release was observed, after 120 min of Cd²⁺ treatment, which is in agreement with the well known half life of the storage compartment of DA in the striatum (Javoy and Glowinski, 1971). Thus reducing extracellular Ca²⁺ impaired simultaneously the Ca²⁺-dependent DA releasing mechanism and the DA synthesis. Under these conditions, the *carrier-mediated* mechanism of release appeared as unaffected but DA was released from the intraterminal stores and no more from the newly synthesized compartment.

As a corollary, a forced Ca²⁺ entry could have opposite effects. Indeed a low dose of Ca²⁺ ionophore A23187 (1 μM) slightly increased basal [³H]DOPAC and dramatically potentiated the amphetamine-induced [³H]DA release. The use of a very high dose amphetamine is devoid of physiological significance but allowed to examine the total DA content of DA terminals in vivo. On control rats, 1 mM amphetamine produced a 34 fold increase of extracellular DA and 5.6 fold increase of [³H]DA. One hour after superfusion with A23187, the amphetamine effect was still more pronounced (53 fold increase of DA and 37 fold increase for [³H]DA). The potentiation of the amphetamine-effect by A23187 treatment mainly affected the [³H]DA showing an enrichment of the DA terminals with newly synthesized molecules and strongly suggesting an increased synthesis. This increased synthesis was not affecting the spontaneous DA-release but was able to potentiate the *carrier-mediated* one when it was evoked. Indeed amphetamine releasing action is known to be independent from Ca²⁺ and to involve the *carrier-mediated* mechanism of release (Hurd and Ungerstedt, 1989; Sulzer et al., 1995).

Finally, cytoplasmic DA potently inhibits TH activity, thus an accumulation of the cytoplasmic DA in terminals, could only be rendered possible by a reduced feed-back inhibition by DA on TH. Such a reduction could have occurred as a consequence of TH activation by Ca²⁺-dependent phosphorylations. Indeed, changes in the inhibitory constant (TH_{ki}) for DA was reported to be well correlated with the phosphorylation-states of TH protein (Pasqualini et al., 1994, 1995).

4.2. The Ca²⁺-dependent DA release

The role of Ca²⁺ in the *carrier-mediated* mechanism of release is not simple. It was recently observed that Cd²⁺ ions, but also NMDA, not only impaired the electrically-evoked and Ca²⁺-dependent DA release

but also induced a dramatic activation of the *carrier-mediated* DA release, this last effect being counteracted by GBR12909 (Olivier et al., 1995). The effect of Cd²⁺ was confirmed in the present study and a competition between the two ions was observed since the Cd²⁺ effects were reduced when Ca²⁺ was maintained in the superfusing fluid. The mechanism by which NMDA and Cd²⁺ affect the *carrier-mediated* DA release remains to be elucidated. It is however well documented that the reversal of the uptake mechanism is linked to the reversal of sodium gradient (Amejdiki-Chab et al., 1992; Levi and Raiteri, 1993; Okada et al., 1990). Cd²⁺ as NMDA could be responsible for such an event, the first by inhibiting the Na⁺/K⁺ ATPase (Pal et al., 1993; Rajanna et al., 1990) and the second by inducing a sustained depolarization of nerve terminals as it was reported for the cell bodies (Chergui et al., 1993; Grace and Bunney, 1984).

4.3. Physiological implications

The simultaneous inhibition of DA synthesis and the marked increase in DA release after Cd²⁺ treatment constitutes a paradoxical situation leading to the impoverishment of DA terminal pools. However, such a situation was already described in a synaptosomal preparation after NMDA treatment (Desce et al., 1994). These observations are in line with the already proposed uncoupling between synthesis and the electrically-evoked DA release (Miu et al., 1992). The physiological significance of an uncoupling between the synthesis and the two mechanisms of DA release must be questioned. Indeed the Ca²⁺-dependent release appears not to be related to the synthesis as long as the storage compartment is not affected or can still compensate for the lack of newly synthesized molecules. In contrast the *carrier-mediated* DA release could be more related to the cytoplasmic DA concentration and in turn to the rate of synthesis. This was highlighted by the presently reported increase of amphetamine effects by a previous treatment with calcium ionophore A23187. Though being without effect on the basal release (the both components), an increased intraterminal DA synthesis could result in an increased *carrier-mediated* release when this process is later evoked by amphetamine. Many experimental situations including local superfusion with glutamate and related substances (Leviel et al., 1990) estradiol (Pasqualini et al., 1995) and GnRH associated peptide (Gobert et al., 1992), were reported to increase or decrease the specific activity of the DA released in the striatum without affecting the release by itself. It can thus be proposed that preterminal regulations controlling cytoplasmic DA content (by increasing or decreasing synthesis) could mainly affect the *carrier-mediated* amine release.

In conclusion, our results show that increasing or decreasing calcium entry produces corresponding effects on the DA synthesis in striatal terminals. They evidence that impaired synthesis can be responsible for apparent alterations in the release. However synthesis is not equally involved in the two mechanisms of DA release, poorly affecting exocytosis as long as the storage compartment is unaffected but directly altering the *carrier-mediated* amine release.

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