0026-895X/96/020338-05$3.00/0

MOLECULAR PHARMACOLOGY, 49:338–342 (1996).

Reserpine Inhibits Amphetamine Action in Ventral Midbrain Culture

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Received June 5, 1995; Accepted October 16, 1995

SUMMARY

Although amphetamine releases catecholamines from isolated secretory vesicles, a number of *in vivo* experiments have indicated that the vesicular amine transport blocker reserpine does not block amphetamine-induced release. To address this paradox, we examined the effect of reserpine on amphetamine-induced dopamine release from postnatal ventral midbrain neurons in culture. These cultures provide a preparation in which intracellular, extracellular, and releasable dopamine pools can be measured simultaneously. We found that 1 μM reserpine for 90 min reduced stimulation-dependent dopamine release by >95%. In parallel, reserpine reduced amphetamine-induced dopamine release by >95% compared with cells not exposed to reserpine or by 75% compared with reserpine-treated cultures. This shows that amphetamine acts principally by redistributing dopamine from synaptic vesicles to the cytosol.

The rewarding actions of AMPH are believed to be due to DA release from mesoaccumbens synaptic terminals (1). Although >90% of neuronal DA is sequestered in synaptic vesicles (2) and AMPH releases catecholamines from isolated synaptic vesicles as well as chromaffin granules (3–6), it has not been clearly shown whether AMPH releases vesicular DA from intact neurons. Because reserpine inhibits the vesicular monoamine transporter located in catecholamine-containing neurons (7), leading to depletion of vesicular DA (8), the drug has been used to determine whether vesicles contribute to AMPH-mediated release. Such studies were initiated more than 35 years ago (9) and are now numerous; despite this, the effects of reserpine on AMPH action have remained controversial.

To address this issue, we used postnatal ventral midbrain DA neuron cultures as a model system. Unlike *in vivo* experiments, all of the DA in the system, including the extracellular, intracellular, and stimulation-dependent releasable pools, can be assayed simultaneously. Postnatal cultures have the further advantage that, unlike synaptosomal preparations, the DA neuron terminals are intact. Unlike the whole animal, reserpine can be applied directly. Finally, feedback pathways and their confounding compensatory actions are absent. With this preparation, we investigated whether reserpine depletion of vesicular DA inhibits AMPH-induced DA release.

Materials and Methods

Ventral tegmental area cultures were prepared as previously described (10). For release studies, cultures were switched from culture medium to physiological incubation medium that contained 135 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 200 μM ascorbic acid, and 10 mM HEPES, pH 7.3. High-potassium medium contained 98 mM NaCl, 40 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 200 μM ascorbic acid, and 10 mM HEPES, pH 7.3. Calcium-free medium contained 121 mM NaCl, 3 mM KCl, 10 mM $MgCl_2$, 10 mM glucose, 10 mM EGTA, 200 μM ascorbic acid, 20 μM pargyline, and 10 mM HEPES, pH 7.3. DA in the medium was measured by electrochemical detection with a Coulochem II high performance liquid chromatograph using Cataphase II mobile phase (ESA, Bedford, MA). To measure intracellular DA concentrations, the medium was removed, 0.3 M perchloric acid was added, the cells were scraped off of the substrate, the suspension spun at 16,000 × *g* for 5 min, and the DA in the supernatant was measured. All experiments were performed in triplicate or quadruplicate using sets of sister cultures 1–4 weeks after plating.

Results

Effect of reserpine on stimulation-dependent release. To determine the rate at which reserpine depletes DA

This work was supported by the Parkinson's Disease Foundation (D.S.), the Stanley Scholars Program (C.S.), and National Institutes of Health Grants MH44736 (S.R.) and DA07418 (D.S.). S.R. is a Burroughs Wellcome Scholar in Experimental Therapeutics, and this work was supported in part by a grant from the Burroughs Wellcome Fund.

ABBREVIATIONS: AMPH, *d*-amphetamine; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; DAT, DA transporter; MAO, monoamine oxidase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

from the releasable pool of synaptic vesicles, we incubated sister cultures in physiological incubation medium for a total of 90 min, including 1 μM reserpine for the last 0, 10, 45, or 90 min. Cultures were then stimulated by incubation in high-potassium medium for 3 min, and extracellular DA was measured (Fig. 1A). Control cultures released 570 ± 34 fmol (mean ± standard error of three experiments) of DA (equivalent to 5.7 ± 0.3 nM) into the medium. Neither DOPAC nor homovanillic acid was observed (<25 fmol/culture). DA release significantly decreased with length of reserpine exposure ($p < 0.0001$ by analysis of variance); reserpine for 90 min reduced DA release to 15 ± 8 fmol/culture (equivalent to 0.1 ± 0.1 nM) or 2.6% of the control. Extracellular DOPAC was observed only after the 10-min reserpine incubation, where it was present at 52 ± 20 fmol/culture (equivalent to 0.5 ± 0.2 nM).

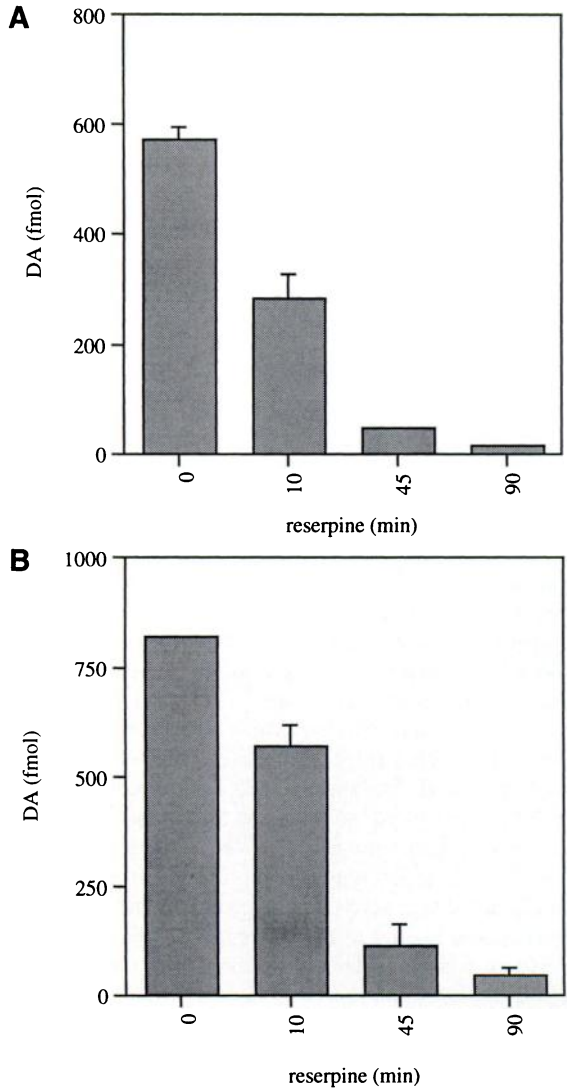

Fig. 1. Reserpine inhibits stimulation-dependent DA release. A, A set of sister midbrain cultures (29 days *in vitro*) were incubated in physiological medium for a total of 90 min, during which 1 μM reserpine was included for the periods indicated. The medium was then replaced with high-potassium medium for 3 min, after which extracellular DA was measured. B, After removal of the medium, the same set of cultures were extracted and analyzed for intracellular DA. The extent of depletion of released and intracellular DA was similar.

To measure the effects of reserpine on whole tissue rather than on the releasable pool of synaptic vesicles, we extracted the cultures and determined intracellular DA concentrations after the high-potassium stimulation. Reserpine produced a time-dependent decrease in DA (Fig. 1B). After 90 min of incubation, control cultures exposed to physiological media alone contained 817 ± 0 fmol/culture of intracellular DA (three experiments). Reserpine (1 μM for 90 min) significantly reduced intracellular levels to 45 ± 23 fmol/culture ($p < 0.0001$ by analysis of variance) or 5.5% of control. There was no difference in the intracellular DOPAC levels between controls and reserpine treatment (controls, 90 ± 13 fmol/culture; 90 min reserpine exposures, 90 ± 26 fmol/culture; two experiments). Together these results indicate that reserpine depletes most cellular DA and that the DA is primarily stored in vesicles.

Effect of reserpine on AMPH-induced DA release. Because we have noted that there is a significant amount of spontaneous activity in DA neuron cultures (10), we used a calcium-free medium to test whether reserpine inhibits AMPH-induced DA release. We also included 10 μM pargyline to eliminate the confound of AMPH inhibition of MAO. This treatment abolished DOPAC in preliminary trials (not shown). Cultures underwent a 90-min incubation with or without reserpine in the calcium-free/pargyline medium followed by a 40-min incubation, from which DA release was measured. We used the concentration of 10 μM AMPH used in previous *in vitro* experiments (11, 12); in agreement with previous reports, we found that 1 μM AMPH does not release detectable DA from midbrain culture (13) (data not shown). Control cultures received neither reserpine nor AMPH. Reserpine cultures received reserpine (1 μM) in both incubations. AMPH cultures received AMPH (10 μM) for the second incubation. AMPH/reserpine cultures received reserpine in the first incubation and both reserpine and AMPH in the second. The baseline extracellular DA level in control cultures was 786 ± 53 fmol, equivalent to 7.9 ± 0.5 nM (three experiments; Fig. 2A). AMPH cultures showed a 250% increase in extracellular DA, to 1958 ± 135 fmol (four experiments), which is equivalent to 20 ± 1 nM. In AMPH/reserpine cultures, extracellular DA was 819 ± 75 fmol (four experiments), which was significantly different from AMPH cultures ($p < 0.001$ by Student-Newman-Keuls test) but not significantly different from control cultures. Reserpine cultures showed reduced extracellular DA levels (431 ± 21 fmol, four experiments). Therefore, although reserpine inhibited nearly all AMPH-induced release, AMPH released a relatively small but significant amount of DA after depletion of the releasable vesicular pool ($p < 0.05$ by Student-Newman-Keuls test).

In the same cultures, we also measured the effects of reserpine and AMPH on intracellular DA. Reserpine (1 μM reserpine for 90 min) decreased cellular DA content to 6.4 ± 2.7% of control (Fig. 2B), which is consistent with the extent of DA depletion in Fig. 1B. AMPH increased the total amount of DA in the cultures (total intracellular plus extracellular levels; Fig. 2C; $p < 0.01$ for cultures without reserpine; $p > 0.05$ for reserpine-treated cultures; Student-Newman-Keuls test). The increase in intracellular DA was not due to MAO inhibition because pargyline was included in all of the incu-

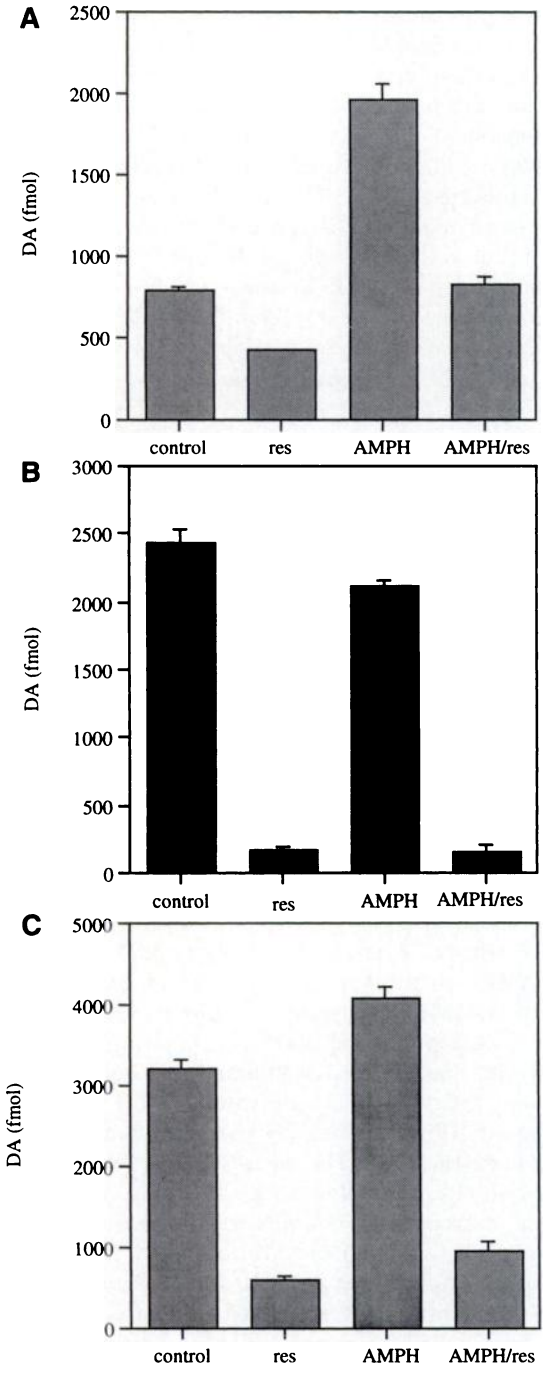

Fig. 2. Reserpine inhibits AMPH-induced DA release. A, A set of sister midbrain cultures (8 days *in vitro*) were incubated in physiological medium with or without 1 μM reserpine (*res*) for a total of 90 min. The medium was then replaced with calcium-free medium for 40 min with or without reserpine as before and with or without 10 μM AMPH. The medium was then removed and analyzed for DA. B, After the removal of the medium, the same set of cultures were extracted and analyzed for intracellular DA. C, Combined intracellular and extracellular DA levels indicate that reserpine profoundly reduced the DA content of the entire system, whereas AMPH induced a small but significant increase in total-system DA.

bation media and neither intracellular nor extracellular DOPAC was observed (i.e., intracellular and extracellular levels were <25 fmol/culture). This experiment was performed three times with similar results.

Discussion

The controversy surrounding reserpine effects on AMPH action derives mainly from *in vivo* experiments with results that have shown little or no effect of reserpine on AMPH-mediated release (14–17), a blockade of AMPH-mediated release by reserpine (18–20), or a combination of both (21, 22). Attempts to resolve this issue using synaptosomes as a model preparation have given contradictory results (11, 12). We suggest that postnatal DA cell cultures provide the more intact system required to examine reserpine effects on AMPH action while avoiding pitfalls of *in vivo* experiments. Our results show that most AMPH-induced DA release derives from reserpine-sensitive stores, i.e., from vesicles. Unlike previous studies, the time dependence of reserpine action was determined for both intracellular and released DA. This showed that reserpine exposures of 90–120 min depleted most vesicular DA and contrasts with the 24-hr exposure that is typical in such experiments. The shorter period reduces the time during which reserpine may induce confounding secondary effects, including the up-regulation of tyrosine hydroxylase expression that occurs at 24 hr (23), whereas the lack of feedback pathways eliminates other potential experimental confounds, such as increased stimulation of midbrain neurons by cortical or ventral pallidal inputs responding to decreased DA transmission in these areas. If, as in most previous studies, AMPH-mediated release in the presence and absence of reserpine is compared with release in control cultures, reserpine reduces release by 10 μM AMPH by >95%. This is identical to the decrease in stimulation-dependent release. If AMPH-mediated release in the presence and absence of reserpine is compared with extracellular DA levels in cultures exposed to reserpine and not AMPH, reserpine reduces release after 10 μM AMPH by 75%.

Note that although sister cultures derived from the same animals give quite similar results (as shown by the standard error values in the figures), different sets of sister cultures have significantly different levels due to age of culture, dissection, or other conditions. Therefore, we only compared within groups of sister cultures. Even with MAO inhibition, we find that reserpine rapidly depletes nearly all DA in the system. Under these conditions, the DA metabolites homovanillic acid and DOPAC were not increased to a level commensurate with the DA depletion. Therefore, either DA was redistributed into the extracellular medium during the initial 90-min reserpine exposures and removed before exposure to high-potassium medium (Fig. 1) or to calcium-free medium (Fig. 2) or DA was metabolized to products that were electrochemically inactive or migrated in the solvent front.

The reported failure of reserpine to inhibit AMPH-induced DA release has provided the primary support for the exchange diffusion model of AMPH action. This mechanism is predicated on the "simple transport model" of the DAT as a recirculating carrier tightly coupled to ion cotransport. Such a carrier would have a substrate binding site that shuttles back and forth between the extracellular and intracellular faces of the membrane. High levels of extracellular substrate such as AMPH would increase the proportion of inward-facing binding sites favoring cytosolic DA efflux on a one-for-one exchange basis. Although AMPH releases DA from cell lines transfected with catecholamine transporters that do not contain the vesicular monoamine transporter, the amount

released is relatively low (24–26) and is increased both in terms of dose-response (>200% of control levels) and duration (>400%) by cotransfection with the vesicular transporter (24). Moreover, in HEK 293 cells reconstituted with the γ-aminobutyric acid uptake transporter (27), net current is detected at substrate gradients that favor either influx or efflux, inconsistent with a one-for-one exchange of substrate across the membrane, which should produce zero net current. Furthermore, if there were recirculating binding sites, there would be current flux in a zero-*trans* state with sodium and chloride on only one side of the membrane; however, no transport-associated current is seen unless the ions are on both sides of the membrane. Although exchange diffusion may be incompatible with the mode of transporter action, reverse transport of DA clearly occurs and is the ultimate mediator of DA release in AMPH action (28). DA efflux could be triggered either by increased cytosolic DA levels due to efflux from synaptic vesicles (28) or by increased intracellular sodium, chloride, or substrate after AMPH uptake, maintaining the transporter in a conformation favoring efflux. Because AMPH is a substrate for the DA uptake transporter, the transporter should contribute to intracellular AMPH accumulation. Also, the MAO inhibition of AMPH may increase cytosolic DA.

In agreement with many studies, we find that some DA release persists after reserpine treatment. We are aware of three factors that could provide a basis for this observation. (i) AMPH could directly release cytosolic DA via exchange diffusion or other mechanisms. (ii) The reserpine exposure we used is insufficient to completely eliminate vesicular DA. (iii) Our experimental conditions increased intracellular DA. Regarding the first possibility, AMPH could release cytosolic DA due to specific AMPH uptake by the DAT (29). According to the exchange diffusion model (30), AMPH could increase shuttling of a carrier protein from the extracellular face to the cytosol, thereby increasing DA efflux. Alternatively, because the free energy for substrate accumulation by the DAT is due to ion cotransport, AMPH-mediated reduction of sodium and chloride gradients across the plasma membrane due to specific AMPH uptake would favor reverse transport. Moreover, as a competitive DAT inhibitor, AMPH would contribute to extracellular DA levels simply by inhibiting reuptake. It should be noted that the amount of DA released by AMPH in the presence of reserpine is small.

Regarding the second possibility, although reserpine depleted stimulation-dependent DA release, it is possible that significant DA remains in a nonreleasable vesicular pool. Our findings confirm that AMPH reduces the average number of DA molecules released per vesicle (28) as well as AMPH-mediated inhibition of stimulation-dependent release (31, 32). Regardless of whether incomplete reserpine effects underlie the current findings, incomplete reserpine action could explain contradictory effects seen with *in vivo* reserpine administration; e.g., subcutaneous administration is more effective than intraperitoneal administration (12). The time allowed for reserpine action may account in part for discrepant findings.

Finally, DA release in the presence of reserpine could be explained by an increase in DA in the total system after AMPH exposure. AMPH increases DA levels by stimulating synthesis (33) and inhibiting DA metabolism by MAO (34). Therefore, the importance of using MAO inhibitors for examining the effect of reserpine on AMPH action has been noted by several groups (14, 19, 32, 35). Because we included a MAO inhibitor, DOPAC levels in all experimental groups were low and MAO inhibition would not explain the increase in total-system DA. A reason to consider alterations in DA synthesis is that although there are conflicting reports on DA release, all relevant studies of which we are aware show that reserpine blocks AMPH-induced norepinephrine release (9, 22, 32, 36). As suggested by Parker and Cubeddu (19), this may be due to continuing DA synthesis, whereas norepinephrine synthesis is blocked because dopamine-β-hydroxylase is intravesicular and thus is dependent on DA uptake into storage vesicles, which would be inhibited by AMPH. This suggests that *de novo* synthesis could underlie much of the reserpine-insensitive AMPH-induced release of DA in studies where it has been observed. An important question that remains unanswered is whether *de novo* synthesis plays a prominent role in AMPH-mediated release in the absence of reserpine.

Acknowledgments

We thank Dr. Serge Przedborski for comments on the manuscript and Dr. Eric Schwartz for discussion of his work on the mode of action of the γ-aminobutyric acid uptake transporter.

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