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INDIRECT AND DIRECT INHIBITION OF DOPAMINE β-HYDROXYLASE BY AMPHETAMINE IN STORAGE VESICLES AND SYNAPTOSOMES

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1 To elucidate the mechanism by which amphetamine produces a functional inhibition of dopamine β-hydroxylase (DBH), the actions of amphetamine on amine uptake and β-hydroxylation were examined in isolated adrenal storage vesicles and in whole brain synaptosomes of the rat.

2 Amphetamine produces a competitive inhibition of catecholamine incorporation into adrenal vesicles with a Ki of 279 μM; this action of the drug accounted for slightly less than half of the total inhibition of β-hydroxylation in the vesicles, indicating that the interference by amphetamine of access of substrate to the enzyme compartment plays an important role in functional inhibition of DBH. The remainder of the effect in vesicles probably represents direct inhibition of the enzyme.

3 In synaptosomes, similar actions on uptake of substrate and on functional enzyme activity were noted, indicating that amphetamine-induced inhibition of the neuronal amine pump can also affect DBH activity if the substrate is supplied exogenously.

4 In addition to the effects of amphetamine *in vitro*, chronic administration of amphetamine to rats produced an increase in total activity of adrenal DBH.

5 The net effect of amphetamine on DBH activity thus represents the summation of direct inhibition of the enzyme, indirect inhibition via reduced access of substrate and enhancement of activity via trans-synaptic induction.

Introduction

Among the many actions of amphetamine on sympathetically innervated tissues is the ability of the drug to produce a functional inhibition of dopamine β-hydroxylase (DBH) both *in vitro* (Goldstein & Contrera, 1962) as well as *in vivo* (Stolk, 1975). Because DBH is contained almost entirely within the adrenergic storage vesicles (Stjärne & Lishajko, 1967), Viveros, Arqueros, Connett & Kirshner, 1969), it is difficult to identify the specific site for this functional inhibition. Amphetamine undoubtedly causes direct inhibition of DBH (Goldstein & Contrera, 1962), but in systems in which the vesicles remain intact, blockade of the vesicular uptake mechanism by amphetamine may produce indirect inhibition by preventing access of dopamine to the enzyme (Slotkin & Kirshner, 1971; Slotkin, Ferris & Kirshner, 1971). Additionally, if β-hydroxylation is measured in synaptosomal preparations or *in vivo* by administration of a radioactive substrate, then the precursor amine must first be taken up into the intraneuronal extravesicular space, and amphetamine also inhibits this process (Coyle & Snyder, 1969; Horn, Coyle & Snyder, 1971). Thus, in a number of studies, the specific actions of amphetamine on DBH activity *in vivo* have been difficult to interpret (Baird & Lewis, 1964; Taylor & Snyder, 1971; Stolk, 1975).

In the present experiments, the actions of amphetamine in lysed or intact adrenal storage vesicles and in synaptosomes are compared in order to determine which of the three factors (direct inhibition of the enzyme, blockade of synaptosomal uptake, blockade of vesicular uptake) play major roles in functional inhibition of DBH activity.

Methods

Experiments in vivo

Male Sprague-Dawley rats (200–250 g, Zivic-Miller) were given (+)-amphetamine sulphate (5 mg/kg, i.p.) every 12 h for 10 days, while controls received 0.9% w/v NaCl solution (saline). Twelve hours after the last injection, the animals were decapitated, and the adrenal glands were homogenized (glass-to-glass) in 2.5 ml of 0.3 M sucrose, buffered at pH 7.4 with 0.025 M Tris-sulphate, containing 10^{-5} M iproniazid; 0.1 ml of the homogenate was taken for catecholamine

analysis (Merrills, 1963) and 0.5 ml of the homogenate, added to 0.5 ml of water, was used for duplicate assays of DBH activity (Friedman & Kaufman, 1965); *p*-hydroxymercuribenzoate (optimal concentration: 0.5 mM) was added to the DBH incubation mixture to inactivate endogenous inhibitors, and [^{3}H]-tyramine (10^{-5} M) was used as the substrate.

The remaining homogenate was centrifuged at 800 *g* for 10 min, and two 0.5 ml portions were used as a source of storage vesicles for uptake determinations (*vide infra*). The rest of the 800 *g* supernatant was centrifuged at 26,000 *g* for 10 min and duplicate 0.1 ml portions withdrawn for assays of tyrosine hydroxylase activity (Waymire, Bjur & Weiner, 1971) using [^{14}C]-tyrosine (10^{-4} M) as the substrate.

Uptake of [^{3}H]-adrenaline into isolated adrenal storage vesicles from control and amphetamine-treated rats was evaluated as described previously (Slotkin & Kirshner, 1973a); 0.5 ml of the 800 *g* supernatant was added to 0.5 ml of sucrose-Tris containing ATP-Mg^{2+} (5 μmol), [^{3}H]-adrenaline (3 μCi) and unlabelled adrenaline (0.1 μmol; to obviate differences in extravesicular catecholamine concentrations among samples). The samples were incubated for 30 min at 30°C while duplicate tubes were kept on ice to serve as blanks. The labelled vesicles were centrifuged 10 min at 26,000 *g*, washed twice, lysed and analyzed for radioactivity and catecholamines. Under these conditions, labelling occurs solely in storage vesicles despite the presence of contaminant organelles (Slotkin & Kirshner, 1973b). The results were expressed as the temperature-dependent component of uptake per gland.

Experiments in vitro

Adrenal glands from untreated rats were homogenized in sucrose-Tris-iproniazid and portions were diluted with water to lyse the vesicles as described above; DBH activity in the lysed preparations was measured in the presence and absence of 1 mM amphetamine using [^{3}H]-tyramine (10^{-4} M) as substrate. The remaining homogenate, containing intact vesicles, was incubated and washed as described for the [^{3}H]-adrenaline uptake studies, except that the incubation mixture contained ATP-Mg^{2+} (5 μmol), fumarate (1 μmol), ascorbate (1 μmol), [^{3}H]-tyramine (5 μCi) and unlabelled tyramine (0.1 μmol), with and without amphetamine. The washed, labelled vesicles were analyzed both for tyramine uptake and for their content of the β-hydroxylated product, octopamine (Slotkin & Green, 1975).

To evaluate the effects of amphetamine on [^{3}H]-adrenaline uptake *in vitro*, the vesicle-containing 800 *g* supernatant from adrenal glands of untreated rats was incubated in the presence and absence of amphetamine (0.5 mM) as described, except that the amount of unlabelled adrenaline added to the medium ranged from 0.01–0.04 μmol; labelled vesicles were then washed and analyzed for catecholamines and radioactivity. Since the vesicular uptake system appears to follow saturation kinetics (Slotkin, 1975), results are reported as double reciprocal plots, enabling calculations of the Km for adrenaline and Ki for amphetamine.

The effects of amphetamine on catecholamine efflux from isolated adrenal storage vesicles were evaluated as described previously (Green & Slotkin, 1973). Vesicle-containing 800 *g* supernatants from untreated rats were pooled and incubated at 30°C for periods ranging from 5 to 60 min in the presence and absence of amphetamine (1 mM). Efflux was stopped by the addition of ice-cold sucrose-Tris, and samples were centrifuged at 26,000 *g* for 10 min and supernatants and pellets analyzed for catecholamines. The percentage of amine remaining in the vesicles was calculated as described previously (Slotkin *et al.*, 1971).

To determine the actions of amphetamine in intact synaptosomes, whole brains from untreated rats were homogenized in 9 vol of sucrose-Tris-iproniazid, centrifuged at 1000 *g* for 10 min, and the supernatant centrifuged at 11,000 *g* for 20 minutes. The crude synaptosomal pellet was resuspended in the same volume of modified Krebs-Henseleit medium containing iproniazid (1.25×10^{-6} M) and ascorbate (2×10^{-6}M) and recentrifuged. The washed pellet was resuspended by gentle homogenization (Teflon to glass) in half the original volume of Krebs-Henseleit medium and 0.5 ml portions were added to 0.5 ml of medium containing 5 μCi of [^{3}H]-tyramine (0.75 nanomole). Samples were incubated for 5 min at 37°C in the presence and absence of varying concentrations of amphetamine, while duplicate tubes were kept on ice to serve as blanks. Tyramine uptake was stopped by the addition of 2 ml of ice-cold Krebs-Henseleit medium and the labelled crude synaptosomes were sedimented at 11,000 *g*, washed and recentrifuged twice and analyzed for radioactivity and octopamine synthesis as described for adrenal vesicles. Centrifugation of the labelled crude fraction in sucrose density gradients (Bosmann & Hemsworth, 1970) indicated that the [^{3}H]-tyramine was indeed taken up primarily into synaptosomes.

Statistics

Data are reported as mean ± s.e. mean and levels of significance are calculated by Student's *t* test (Wine, 1964). Uptake kinetics are presented as double-reciprocal plots (Lineweaver & Burk, 1934); the slopes, intercepts and standard errors are calculated by the method of least squares (Wine, 1964).

Materials

(±)-Adrenaline-[7-^{3}H], tyramine-[G-^{3}H] and (−)-tyrosine-[1-^{14}C] were purchased from New England Nuclear Corp. Iproniazid phosphate, tyramine hydrochloride and (+)-amphetamine sulphate were obtained from Sigma Chemical Corp., and (−)-adrenaline bitartrate from Winthrop Laboratories.

Results

The chronic administration of amphetamine (5 mg/kg) produced little or no alteration in either adrenal catecholamine concentrations or in the ability of isolated adrenal storage vesicles to incorporate [^{3}H]-adrenaline (Table 1). However, the activities of both tyrosine hydroxylase and dopamine β-hydroxylase (DBH) were elevated substantially (130% and 40% above controls, respectively).

In contrast, direct addition of amphetamine to the medium containing the adrenal vesicles produced effective inhibition of uptake (Figure 1). The effect appeared to be competitive, with a Ki for amphetamine about 10-fold higher than the Km for adrenaline. Since vesicular uptake represents the summation of two processes, inward transport minus efflux (Slotkin, 1975), it was important to establish whether the inhibitory effect of amphetamine represented a reduction in transport or an acceleration of efflux. Amphetamine *in vitro* had no effect on the rate of loss of catecholamines from the vesicles (Figure 2), indicating that the blockade of uptake probably results from an effect on inward transport.

Schneider (1972) has reported that amphetamine releases catecholamines from bovine isolated adrenal vesicles; the release is of a lytic nature, requiring the presence of salts (Ferris, Viveros & Kirshner, 1970).

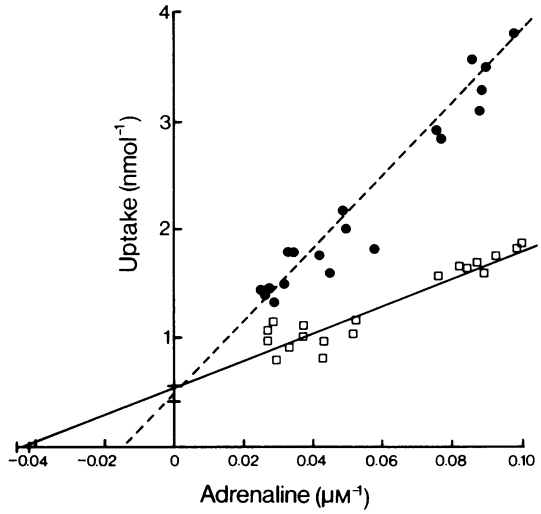

Figure 1 Lineweaver-Burk plot of adrenaline uptake into isolated rat adrenal medullary storage vesicles in the presence (●) and absence (□) of amphetamine (0.5 mM). Intercepts on the ordinate scale are significantly different ($P<0.001$), while intercepts on the abscissa are not. For adrenaline uptake without amphetamine, Km = 23 ± 1 μM and maximal uptake = 1.80 ± 0.08 nmol per gland; with amphetamine, Km = 64 ± 6 μM and maximal uptake = 2.02 ± 0.18 nmol per gland. Ki calculated for amphetamine = 279 ± 28 μM. Lines are drawn by least squares analysis.

In the present study efflux was measured in sucrose, which leads to release via leakage of amines across intact vesicle membranes (Slotkin *et al.*, 1971) rather than lysis, and amphetamine did not alter this efflux.

Table 1 Effects of chronic amphetamine administration (5 mg/kg i.p., every 12 h for 10 days) on the rat adrenal medulla

Determination	*Control*	*Amphetamine*
Catecholamines (μg/gland)	8.91 ± 1.16	9.68 ± 0.78
[^{3}H]-Adrenaline uptake (nmol/30 min per gland)	2.15 ± 0.16	2.13 ± 0.21
Tyrosine hydroxylase (nmol $^{14}CO_2$ formed/h per gland)	14.9 ± 0.7	33.9 ± 2.8*
Dopamine β-hydroxylase (nmol [^{3}H]-octopamine formed/h per gland)	1.73 ± 0.08	2.47 ± 0.32*

Activities were measured *in vitro* after administration of amphetamine or saline *in vivo*. Each group consisted of six rats.
* $P<0.001$ vs. control

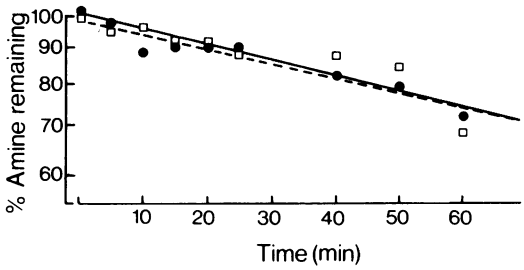

Figure 2 Efflux of endogenous catecholamines from isolated adrenal medullary vesicles in the presence (●) and absence (□) of 1 mM amphetamine, represented as percentage of amine remaining in the vesicles. Lines are drawn by least squares analysis; ordinate scale is logarithmic.

The ability or inability of amphetamine to cause accelerated loss of catecholamines thus probably depends upon the medium used.

Since many other amines besides adrenaline can utilize the vesicular transport system (Slotkin & Kirshner, 1971), it seemed likely that uptake-related effects of amphetamine on DBH could be evaluated by use of a DBH substrate which relies on uptake to gain access to the enzyme. One such amine is tyramine (Slotkin & Kirshner, 1971; Slotkin, 1975; Slotkin & Green, 1975). When amphetamine was added to a preparation of intact adrenal storage vesicles, the uptake of [^{3}H]-tyramine was reduced by 20% (Table 2). However, the net inhibition of β-hydroxylation was much larger (50%). Lysis of the vesicles prior to incubation produced a marked enhancement of [^{3}H]-octopamine synthesis compared

Table 2 Effects of amphetamine *in vitro* on tyramine uptake and octopamine synthesis in intact adrenomedullary vesicles, and on dopamine β-hydroxylase activity in lysed vesicles

Determination	*Control*	*Amphetamine (1 mM)*	*% inhibition*
Intact Vesicles			
[^{3}H]-Tyramine uptake (nmol/30 min per gland)	0.714 ± 0.014	0.567 ± 0.008*	21 ± 1
[^{3}H]-Octopamine synthesis (nmol/30 min per gland)	0.316 ± 0.006	0.159 ± 0.002*	50 ± 1
Lysed Vesicles			
[^{3}H]-Octopamine synthesis (nmol/h per gland)	12.6 ± 0.2	6.07 ± 0.10 *	52 ± 1

Intact vesicle preparations were incubated 30 min with [^{3}H]-tyramine (0.1 mM) and values are reported for that period; lysed vesicles were incubated for 60 min with [^{3}H]-tyramine (0.1 mM). Seven determinations were done for each point.
* $P < 0.001$ vs. control

Table 3 Effects of amphetamine *in vitro* on tyramine uptake and octopamine synthesis in crude whole brain synaptosomes

	Amphetamine concentration		
Determination	*0*	*10^{-6} M*	*10^{-5} M*
[^{3}H]-Tyramine uptake (nmol/5 min per g of brain)	0.137 ± 0.004	0.112 ± 0.001†	0.084 ± 0.003†
% inhibition	—	18 ± 1	39 ± 2
[^{3}H]-Octopamine synthesis (nmol/5 min per g of brain)	0.024 ± 0.001	0.020 ± 0.001*	0.013 ± 0.0004†
% inhibition	—	17 ± 4	46 ± 2

Seven determinations were done for each point. [^{3}H]-Tyramine concentration in the medium was 7.5×10^{-7} M
* $P < 0.02$ vs. control.
† $P < 0.001$.

to the intact preparation; amphetamine produced slightly greater than 50% inhibition in the lysed vesicles.

Since tyramine also has an affinity for uptake into adrenergic neurones which is comparable to that of the natural catecholamine substrates (Burgen & Iversen, 1965), this compound was used also to determine the relative contribution of uptake inhibition by amphetamine to functional inhibition of DBH in crude synaptosomes prepared from whole brain homogenates. In the absence of amphetamine, approximately one-fifth of the [^{3}H]-tyramine taken up was converted to [^{3}H]-octopamine (Table 3). With increasing amphetamine concentrations, there was a progressive decline in both uptake and β-hydroxylation.

Discussion

The enclosure of dopamine β-hydroxylase (DBH) within a membrane-bound particle, the catecholamine storage vesicle, makes it possible to study the indirect inhibition of the enzyme, *i.e.* a reduction in activity by preventing access of substrate to the site of the enzyme. In situations where activity is measured in intact tissues or intact organelles, the uptake of substrate can be rate-limiting (Laduron, 1975); under these circumstances lysis of the vesicles should produce a large increase in activity, as illustrated in Table 2. It was therefore important to assess the degree to which amphetamine could inhibit uptake of substrate and whether the resulting indirect inhibition of DBH could account for a substantial proportion of the net effect of the drug. In isolated adrenal medullary vesicles incubated with amphetamine, there was a competitive inhibition of the catecholamine uptake system; the Ki for amphetamine was approximately the same as that obtained previously for other phenethylamine derivatives (Slotkin, Anderson, Seidler & Lau, 1975). The lack of effect of amphetamine on vesicular catecholamine efflux and the competitive nature of inhibition indicate that the site of action is probably the ATP-Mg^{2+}-stimulated transport system in the storage vesicle membrane (Slotkin, 1973, 1975).

Despite the ability of amphetamine to inhibit uptake of substrate into the vesicles, not all of the functional inhibition of DBH could be accounted for on the basis of reduced access; while vesicular uptake of tyramine was reduced by 20% by amphetamine, conversion to octopamine fell by 50%. These data suggest that even in intact organelles a substantial proportion of DBH inhibition by amphetamine results from a direct effect on the enzyme (Goldstein & Contrera, 1962; Stolk, 1975).

In contrast to the effects of amphetamine *in vitro*, administration of the drug to rats did not affect subsequently determined adrenal vesicular uptake of ^{3}H-adrenaline and actually increased adrenal DBH activity. The differences result from the reversible nature of amphetamine's effects in these systems (Stolk, 1975); because uptake and DBH activity were measured in adrenal homogenates, the inhibition by amphetamine was reversed during tissue preparation as the drug was washed out. On the other hand, functional inhibition of DBH by amphetamine has been observed readily after administration of substrate *in vivo* (Stolk, 1975). The increase in adrenal DBH and tyrosine hydroxylase activities after chronic administration probably represents trans-synaptic induction resulting from amphetamine-induced stimulation of the sympatho-adrenal axis (Mandell & Morgan, 1970; Koda & Gibb, 1973).

In addition to inhibiting amine uptake into storage vesicles, amphetamine blocks amine transport across the neuronal membrane (Coyle & Snyder, 1969; Horn *et al.*, 1971). Thus, in an intact organelle (synaptosome), the same indirect effect of amphetamine should be observed. In the current study, concentrations of amphetamine which inhibited tyramine uptake by 40% produced equivalent inhibition of β-hydroxylation, indicating that blockade of transport contributes substantially to inhibition of DBH. The amphetamine-induced reduction in uptake of DBH substrates from extracellular to intracellular sites is of questionable significance *in vivo* because under normal circumstances, dopamine is synthesized intracellularly and thus must be translocated only from the cytoplasm to the vesicle; however, in situations in which DBH activity is measured by administration of an exogenous substrate, inhibition of neuronal uptake by amphetamine can be a major factor in the net effect.

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