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Lack of Epinine Formation in Adrenal Medulla and Brain of Rats during Cold Exposure and Inhibition of Dopamine β-Hydroxylase

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Summary. Cold exposure of rats for 4 h and simultaneous inhibition of dopamine β-hydroxylase by FLA-63 (25 mg/kg) led to a reduction of the catecholamine content of the adrenal medulla by 46% and of the brain by 68%. Additional injections of 5 mg/kg FLA-63 4 and 9 h after beginning of the experiments, respectively, kept the catecholamine content on this low level (brain) or decreased it further (adrenal medulla). Administration of 5 mg/kg (-)DOPA together with the mono-amine oxidase inhibitor pargyline (50 mg/kg) 24 h after the first injection of FLA-63 stimulated the resynthesis. It amounted for the adrenal medulla to 20 µg/kg body weight/8 h and for the brain to 45 ng/g tissue wet weight/8 h. Paper chromatographic analyses of the extracts of adrenal medulla and brain, respectively, performed at each time of the different injections, clearly identified adrenaline, noradrenaline and dopamine (in traces) in the adrenal medulla as well as noradrenaline and dopamine in the brain; epinine on the contrary could not be demonstrated, not even in traces. Since at least 25 ng of epinine can be detected with certainty by our method, it can be concluded that epinine is not formed in amounts greater than 75 ng/pair adrenal glands or 37.5 ng/brain. The present results support the view that the main pathway of adrenaline biosynthesis in the suprarenal medulla and the brain proceeds via noradrenaline and not via epinine.

Key words: Biosynthesis of catecholamines – Epinine – Brain – Adrenal medulla – Inhibition of dopamine β-hydroxylase.

INTRODUCTION

According to the classical concept, first proposed by Blaschko (1939) and Holtz (1939), the biosynthesis of the catecholamines – e.g. in the suprarenal medulla – proceeds from tyrosine via (-)DOPA to dopamine. The latter enters the catecholamine storing granules, where it is converted to noradrenaline by dopamine β-hydroxylase (DBH), which is localized in these granules (Laduron and Belpaire, 1968; Kirshner, 1962). Noradrenaline has to be released into the cytoplasm in order to be methylated to adrenaline. Then it is taken up into the storage granules. The enzyme responsible for the last step of this biosynthetic pathway, phenylethanolamine-N-methyltransferase (PNMT), has been extensively studied by Axelrod (1962). He showed that PNMT is highly specific towards phenylethanolamine derivatives. S-Adenosyl-L-methionine (SAM) is, as generally considered, the methyl-donor for this N-methylation reaction (Kirshner and Goodall, 1957).

Recently, Laduron (1972) proposed an alternative pathway for the biosynthesis of adrenaline. He postulated, that PNMT is not an enzyme of such a high specificity and is able also to methylate dopamine to epinine. Epinine is now transported into the storage granules, where it is converted to adrenaline. This step of N-methylation of non-β-hydroxylated phenylethylamines was described for N-methyltransferases of the bovine adrenal medulla (Laduron, 1972) and of the rat brain (Hsu and Mandell, 1973; Laduron, 1972a). While the N-methyltransferase of the bovine adrenal medulla involves the common methyl donor SAM, the N-methyltransferases of the rat brain require 5-methyltetrahydrofolic acid (5-MTHF) for the methylation of phenylethylamines. The formation of ^{14}C-labeled epinine was also observed during perfusion of the bovine adrenal medulla with ^{14}C-labeled tyrosine, added to the perfusion solution (Laduron et al., 1974).

In contrast to these results, Pendleton and Gessner (1975) observed neither with SAM nor with 5-MTHF as coenzymes a formation of epinine, using a partially

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purified PNMT from the rabbit or bovine adrenal medulla. Similar results were described by Hofman et al. (1975), who showed in experiments with a highly purified bovine PNMT, that noradrenaline has a 125 fold higher affinity to PNMT than dopamine has. Moreover, the incubation of dopamine with 5-MTHF did not result in the production of epinine, but rather in the enzymatic formation of formaldehyde, which condenses nonenzymatically with dopamine to build 6.7-dihydroxy-tetrahydro-iso-quinoline (TIQ) (Meller et al., 1975).

According to these results, obtained by in vitro experiments, the question is still under discussion, whether or not the formation of epinine from dopamine occurs and whether this reaction is an important step in the biosynthesis of adrenaline. We studied, therefore, the possibility to synthesize epinine in intact rats. For this purpose DBH was inhibited by the administration of FLA-63, a well known DBH-inhibitor (Florvall and Corrodi, 1970) and the animals were exposed to cold for 4 h. The deficit of catecholamines induced in this way stimulates the resynthesis, which was further accelerated by the administration of precursors, (-)DOPA and dopamine, respectively. Since the DBH is strongly inhibited, the enhanced synthesis should lead to a preferential formation of epinine, if such a reaction really occurs.

Abbreviations Used. DBH, Dopamine β-hydroxylase; PNMT, phenylethanolamine-N-methyltransferase; SAM, S-adenosyl-L-methionine; 5-MTHF, 5-methyl-tetrahydro-folic acid; TIQ, 6,7-dihydroxy-tetrahydro-isoquinoline; MAO, monoamine oxidase.

MATERIAL AND METHOD

Male Wistar rats (200–250 g) were injected i.p. with 25 mg/kg FLA-63 and kept for 4 h at 4° C. At this time and 5 h later they got 2 further injections of 5 mg/kg FLA-63 and were kept at 22° C throughout. Twenty-four hours after the beginning of the experiments the animals were injected with 5 mg/kg FLA-63, 50 mg/kg pargyline i.p. and 5 mg/kg (-)DOPA s.c., 8 h later they were killed. Their adrenal glands and brains were prepared, weighed and homogenized. At 0, 4, 9 and 24 h after start of the experiments 1 group of 4 rats each was killed and their adrenal glands and brains were taken for analysis.

One pair of adrenal glands was homogenized in 2 ml ice-cold 0.4 N $HClO_4$ with a glass homogenizer; the homogenate was centrifuged with 800 g for 20 min at 4° C. Of the supernatant, 0.5 ml was taken for determination of noradrenaline and adrenaline and the remaining supernatant was used for paper chromatography.

The brains were homogenized in 10 ml ice-cold 0.4 N $HClO_4$ containing 0.1 g Na_2-EDTA with an Ultra-Turrax for 15 s. The homogenates were centrifuged with 1500 g for 30 min in the cold. The supernatant was brought up to pH 4 with solid K_2CO_3, kept for 20 min at 4° C and the precipitated $KClO_4$ was removed by centrifugation. The supernatant of two homogenates were combined, the pH was adjusted to 6.5 with 0.5 M K_2HPO_4-solution and the catecholamines were purified by column chromatography on Dowex 50 WX 4 (Schümann and Grobecker, 1965). The adsorbed catecholamines were eluted first with 10 ml 1 N HCl, followed by 10 ml 1.2 N HCl. Of the 1 N HCl eluate, 2 ml were taken for the noradrenaline determination, the remaining 8 ml as well as the 10 ml of the 1.2 N HCl eluate were used for the chromatographic analysis.

Table 1. R_f-values of catecholamines. 1 µl of each amine solution containing 1 µg were spotted on Schleicher and Schüll paper 2043a and chromatographed as described in Methods. The final migration of the solvent (n-butanol/1 N HCl) front was 42.0 cm from the origin

Amine	R_f
Noradrenaline	0.29
Adrenaline	0.36
Dopamine	0.46
Epinine	0.55

The eluates of 2 pairs of adrenal glands and those of 2 brains, respectively, were evaporated to dryness in vacuo at 52–56° C bath temperature. The residues were redissolved in 0.3 ml (adrenal glands) and 0.2 ml (brains) ethanol/1 N HCl 8:2 (v/v), respectively. 50 µl of the extracts of the adrenal glands or 70 µl of the extracts of the brains were spotted on Schleicher and Schüll paper 2043a and were subjected to descending chromatography in n-butanol/1 N HCl for 24 h under CO_2-atmosphere. The spots were developed according to Elimann (1958) with a 5% solution of ethylenediamine containing 0.1% K_3 [$Fe(CN)_6$]. After drying at 50° C the spots were identified by UV-radiation. In each chromatogram 1 µg (in 1 µl) of noradrenaline, adrenaline, dopamine and epinine were run separately as reference substances. The spots for the 4 amines were clearly separable as shown by the R_f-values, listed in Table 1. Adrenaline and noradrenaline were determined by the trihydroxyindole method (Palmer, 1964).

Drugs were dissolved in NaCl solution (0.9%), except FLA-63, which was dissolved in a small volume of acetic acid, diluted with NaCl solution (0.9%) and neutralized by the addition of sodium acetate buffer (pH 6.0).

Drugs Used. (-)Dihydroxy-phenylalanine [(-)DOPA] (Deutsche Hoffmann La Roche AG, Grenzach); 3-hydroxy-tyramine hydrochloride (dopamine) (Serva, Heidelberg); pargyline hydrochloride (Abbott Laboratories, Chicago); epinine hydrochloride (Boehringer, Mannheim); (-)suprarenine-base (adrenaline) and (-)arterenol base (noradrenaline) (Hoechst, Frankfurt); bis (4-methyl-1-homopiperazinylthiocarbonyl)-disulfide (FLA-63) was kindly supplied by Dr. H. Corrodi (AB Hässle, Göteborg).

Statistical Methods. Significant differences of experimental values were estimated by means of Student's t-test. A P value smaller than 0.05 is considered to be significant.

RESULTS

Influence of Cold Exposure and Inhibition of DBH by FLA-63 on the Catecholamine Content of the Rat Adrenal Gland

Rat adrenal glands contained 131.99 ± 3.43 µg/kg body weight catecholamines ($N = 4$); the percentage degree of noradrenaline amounted to approx. 6%. After injection of 25 mg/kg FLA-63 and cold exposure

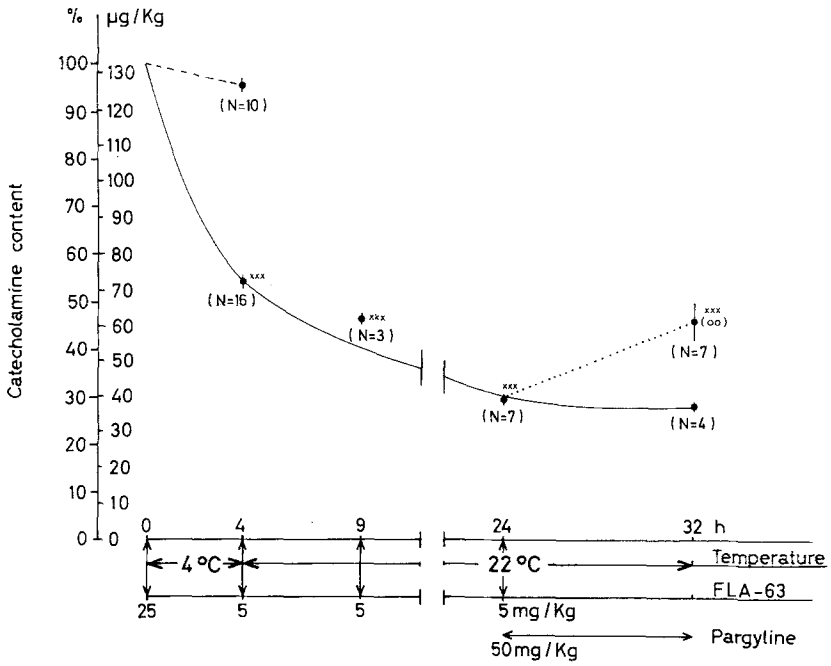

Fig. 1
Decrease of catecholamine content in the rat adrenal medulla after administration of FLA-63 and cold exposure. Rats were exposed to cold for 4 h after i.p. injection of 25 mg/kg FLA-63. Thereafter the animals were kept at 22° C. At 4, 9 and 24 h after onset of the experiments 3 further i.p. injections of 5 mg/kg FLA-63 each were administered and at 24 h in addition 50 mg/kg pargyline i.p. and in some experiments 5 mg/kg (-)DOPA s.c. At 32 h the rats were killed. Ordinate: Catecholamine content in µg/kg body weight or in percent. Control value of untreated animals: 131.99 µg/kg = 100%. Abscissa: Time of drug administration, temperature and drugs. Interrupted line: Catecholamine content without drug treatment. Solid line: Catecholamine content after treatment with the given drugs. Dotted line: Catecholamine content after additional treatment with 5 mg/kg (-)DOPA. Given are means ± S.E.M. N = number of observations. ^{xxx} $P < 0.001$ vs. control; ^{oo} $P < 0.005$ vs. 24 h value

for 4 h at 4° C the catecholamine content was reduced by about 46% (Fig. 1). This decrease is mainly caused by the strong inhibition of the DBH, since the catecholamine content of the control group – i.e. animals treated with 0.9% saline solution and kept for 4 h at 4° C – was not significantly changed. The only change observed in this group was an alteration of the adrenaline/noradrenaline ratio. After the cold treatment the percentage noradrenaline content increased from 6 to about 14%.

After the cold period the animals were kept at room temperature. Two additional injections of 5 mg/kg FLA-63 decreased the catecholamine content by a further 24%. It amounted 24 h after beginning of the experiments to 39.44 ± 1.48 µg/kg, which corresponds to approx. 30% of the original content. At this time, there were administered 5 mg/kg FLA-63 plus 5 mg/kg (-)DOPA in order to stimulate the resynthesis and 50 mg/kg pargyline, which should protect possibly formed epinine from degradation by monoamine-oxidase (MAO). Eight hours after these last injections the catecholamine content rose to 60.30 ± 5.41 µg/kg corresponding to an percentage increase of about 53%; nearly the same increase was obtained, when under equal conditions dopamine instead of (-)DOPA was injected, i.e., the catecholamine content rose to 60.16 ± 1.32 µg/kg (N = 3). The catecholamine content of the control group, on the other hand, pretreated in the same manner but without (-)DOPA or dopamine, remained unchanged at its low level.

Influence of Cold Exposure and Inhibition of DBH by FLA-63 on the Noradrenaline Content of the Rat Brain

The noradrenaline content of the rat brain amounted to 235.23 ng/g tissue wet weight. Cold treatment and injection of 25 mg/kg FLA-63 reduced the noradrenaline content by about 67% (Table 2), while that of the control group remained unchanged. Two further injections of 5 mg/kg FLA-63 did not change the low noradrenaline content, which – 24 h after beginning of the experiments – amounted to 73.49 ± 2.07 ng/g. After simultaneous injections of (-)DOPA, FLA-63 and pargyline, the noradrenaline content increased during the following 8 h to 115.89 ± 5.84 ng/g, while it remained unchanged in the corresponding control group, pretreated only with FLA-63 and pargyline.

Paper Chromatographic Analysis of the Extracts of Rat Adrenal Glands and Brain

The paper chromatographic analysis of the extracts was performed descending with the solvent n-butanol/1 N HCl under CO_2-atmosphere; the spots were developed with 0.1% K_3 [$Fe(CN)_6$] solution and identified by UV-radiation, as described in Methods. With this procedure it was possible to clearly identify 25 ng of each amine present in the spots. The extracts of tissues obtained at each time interval of the different injections were chromatographed. Noradrenaline, adrenaline and traces of dopamine were clearly detect-

Table 2. Influence of cold exposure and of FLA-63 on the noradrenaline content of the rat brain. At 0 time rats were injected i.p. with 25 mg/kg FLA-63 and kept for 4 h at 4° C. Thereafter the rats were kept at 22° C throughout the experimental time. Explanation of the additional administration of the drugs see legend to Figure 1. Given are the means ± S.E.M. of 4 experiments

Time of drug action (h)	Drugs (mg/kg)		Noradrenaline ng/g tissue wet weight	Δ % vs. control
	FLA-63	pargyline		
0	control		235.23 ± 3.66	–
0–4	cold treated control		247.43 ± 3.01 n.s.	+ 5.15 ± 0.99
0–4	25	–	76.84 ± 2.60*	−67.34 ± 0.86
4–9	+5	–	–	–
9–24	+5	–	73.49 ± 2.07*	−68.76 ± 0.68
24–32	+5	50	68.97 ± 1.17*	−70.68 ± 0.39
24–32	+5	50 + 5 (-)DOPA	115.89 ± 5.84*,**	−50.75 ± 1.92

* $P < 0.001$ vs. control; ** $P < 0.001$ vs. 24–32 h without (-)DOPA.
n.s. = not significant vs. control.

ed in the extracts of the adrenal glands as well as noradrenaline and dopamine in those of the brains. However, we were not able to detect any epinine, not even in minimal traces. On the other hand, 25 ng of epinine mixed to the spots of the extracts, were clearly identified.

DISCUSSION

Inhibition of DBH by FLA-63, a strong DBH inhibitor, and cold exposure for 4 h at 4° C reduces the catecholamine content of the rat adrenal glands by about 46% and that of the rat brain by about 67%. The decrease of the noradrenaline content of the rat brain is in accordance with observations of Florvall and Corrodi (1970), while no data existed about the influence of FLA-63 on the adrenal gland. The present results show, that FLA-63 is similar potent in the inhibition of the DBH of the rat adrenal gland than in the brain. The reduced catecholamine content is further decreased by an additional administration of FLA-63 or remains at least on its low level. Since DBH is mainly inhibited, the strong stimulation of the resynthesis, induced by the deficit of the catecholamine content, should lead to the formation of epinine, if this pathway is of importance for the biosynthesis of adrenaline. The present results indicate, that under our experimental conditions a formation of epinine does not occur. According to the sensitivity of the paper chromatographic method one can calculate, how much epinine could be formed without being detectable. The extracts of 2 pairs of adrenal glands were concentrated in 0.3 ml solvent. 50 µl of this solution were spotted on the paper chromatogram. These 50 µl cannot contain more than 25 ng epinine, since we should have been able to detect this amount, as described under results. Therefore, we can exclude that the total extract of two pairs of adrenal glands contains more than 6×25 ng = 150 ng epinine corresponding to 75 ng/pair adrenal glands or 0.3 µg/kg body weight. By similar calculation (cf. Methods) we can also exclude that more than 37.5 ng epinine/brain or 20.2 ng/g brain are formed. After the additional administration of the precursors for noradrenaline, (-)DOPA as well as dopamine, and after the inhibition of MAO the catecholamine content of the adrenal glands rises by about 20 µg/kg during 8 h. Schümann (1958) showed, that the catecholamine content of the rat adrenal glands is decreased by about 74% 9 h after the administration of insulin, which is comparable to the reduction here described, which was brought about by FLA-63 treatment after 24 h. According to Schümann (1958), during the following 15 h the catecholamine content increases by about 50%, which corresponds to a rate of resynthesis of 40 µg/kg/15 h. These observations are in good agreement with the present results, where a resynthesis of 20 µg/kg/8 h was obtained. The results are compatible with the view, that a high dosis of the precursor dopamine – either given as such or as (-)DOPA, which is very quickly converted to dopamine – is able to displace FLA-63 from its binding site at the DBH. Moreover, our results indicate, that all enzymes involved in the biosynthesis of the catecholamines are functioning. However, since during the first 24 h of the experiments these enzymes except DBH are active and the strong inhibition of DBH by FLA-63 prevents the formation of noradrenaline, the enhanced resynthesis, induced by the reduction of the catecholamine content, should preferentially lead to the synthesis of epinine, if such a formation is indeed an important step in the biosynthesis of adrenaline. The present results clearly indicate, that the existence of epinine cannot be demonstrated. This is in accordance with observations of Hoffman et al. (1975), who showed, that PNMT

has a much higher affinity to noradrenaline than to dopamine. Moreover, in vivo experiments in rats strongly indicate (Pendleton and Gessner, 1975), that the time course of the conversion of dopamine to adrenaline is in good agreement with the classical concept, that noradrenaline is the physiological precursor of adrenaline. After the administration of ^{3}H-dopamine, the authors observed during the first 2 h primarily the formation of ^{3}H-noradrenaline, while during this early time only small amounts of ^{3}H-adrenaline are formed. During the following 12 h, however, the amount of ^{3}H-noradrenaline nearly completely disappeared, while simultaneously the amount of ^{3}H-adrenaline is strongly increased. The formation of ^{3}H-epinine, on the contrary, was not detectable.

The N-methyltransferase isolated from rat brain (Hsu and Mandell, 1973; Laduron, 1972a), which was also partially purified (Laduron et al., 1974a), requires 5-MTHF as methyl donor for the methylation of phenylethylamine derivatives. Since 5-MTHF was found to be localized in greater amounts in various regions of the brain (Korevaar et al., 1973), one could expect, that at least in the brain the formation of epinine occurs, especially as the DBH is strongly inhibited. After the administration of 25 mg/kg FLA-63, as shown in Table 2, the noradrenaline content of the brain is reduced to approx. 32% of the original content during 4 h cold exposure and remains unchanged after further injections of 5 mg/kg FLA-63. This is accordance with observations of Florvall and Corrodi (1970), who showed, that 8 h after a single dosis of 25 mg/kg FLA-63 the noradrenaline content of the rat brain is decreased to about 30% of the original content and rises during the next 16 h to approx. 70%. This latter increase as well as the present observation, that the administration of 5 mg/kg (-)DOPA increases the noradrenaline content by about 55%, is a good indication, that under our experimental conditions—and also under those described by Forvall and Corrodi (1970)—all enzymes involved in the biosynthesis of the catecholamines in the rat brain are not reduced in their function. Although optimal conditions for the formation of epinine should exist we could not detect any epinine by paper chromatography.

The distribution of 5-MTHF in various regions of the brain does also not support the view, that 5-MTHF is involved in the biosynthesis of adrenaline. The areas with the highest density of indoleamine cell bodies contain the highest amount of 5-MTHF, while the regions, which are rich in dopaminergic (substantia nigra) and noradrenergic nerve terminals (locus coeruleus) are relatively poor in 5-MTHF (Korevaar et al., 1973).

Moreover, the observation of Meller et al. (1975) contradicts also the postulate of Laduron (1972a), that the N-methyltransferase from the rat brain requires 5-MTHF as methyl donor for the conversion of dopamine to epinine. The authors showed, that dopamine incubated with 5-MTHF reacts enzymatically in the formation of formaldehyde, which condenses nonenzymatically with dopamine to TIQ. TIQ has very similar chromatographic characteristics as epinine and may therefore lead to a misidentification of the reaction products formed during the incubation of dopamine with 5-MTHF.

According to the present results and to the observations of other authors—discussed above—it is very likely, that the biosynthesis of the catecholamines follows the classical concept, that noradrenaline, formed from dopamine, is the physiological precursor for adrenaline. The alternative pathway, postulated by Laduron (1972), cannot definitively be excluded, but may be at best an unimportant side-way.

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