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EFFECT OF RESERPINE AND PROTRIPTYLINE ON THE SUBCELLULAR DISTRIBUTION OF ^{3}H-METARAMINOL IN THE MOUSE HEART

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Exogenously administered metaraminol (MA) (Andén, 1964 ; Shore, Busfield & Alpers, 1964 ; Gram & Wright, 1966) or MA formed by the decarboxylation and subsequent β-hydroxylation of α-methyl-meta-tyrosine (Porter, Totaro & Leiby, 1961 ; Carlsson & Lindqvist, 1962 ; Udenfriend & Zaltzman-Nirenberg, 1962 ; Gessa, Costa, Kuntzman & Brodie, 1962 ; Carlsson, 1964) can accumulate in sympathetically innervated tissues and displace endogenous noradrenaline. MA is rapidly taken up by cardiac tissue and measurable amounts can be found for at least 8 days (Gram & Wright, 1966). The MA taken up is accumulated within adrenergic nerves (Jonsson & Ritzén, 1966), can be released by sympathetic nerve stimulation (Crout, Alpers, Tatum & Shore, 1964) and is not taken up by hearts of immunosympathectomized animals (Shore *et al.*, 1964).

At least part of the MA taken up is bound to subcellular particles obtained from rat (Giachetti & Shore, 1965) or mouse (Lundborg & Waldeck, 1966) hearts. Recent work in our laboratory has suggested the existence of two distinct mechanisms for the incorporation of biogenic amines into adrenal medullary (Lundborg, 1966) and adrenergic nerve granules (Lundborg & Waldeck, 1966 ; Lundborg & Stitzel, 1967), a reserpine-sensitive and a reserpine-resistant uptake mechanism. *In vitro* MA was preferentially taken up by the reserpine-resistant mechanism, but *in vivo* studies have shown that MA could be further incorporated by a mechanism which could be blocked by reserpine. The experiments reported here were performed to examine these two mechanisms in greater detail and to study the possibility of a transfer of amines from one site to the other.

METHODS

In vivo experiments. Mice, divided into groups of 6, were given ^{3}H-MA (100 mc/mM), 40 μg/kg. intravenously. Control groups received no further treatment and were killed by decapitation either 30 min, 60 min or 24 hr later. Other groups were injected with reserpine (0.5 mg/kg, intravenously) or protriptyline (10 mg/kg, intravenously) 15 min or 24 hr after the ^{3}H-MA injection and were killed either 15 or 45 min later. A more detailed presentation of the injection schedules can be found in the Results section. All animals were kept at 30° C.

The hearts were removed and homogenized in the cold using a plastic pestle. The homogenization medium was 0.25 M sucrose containing 0.005 M phosphate buffer, pH 7.4, and 0.001 M $MgCl_2$. A coarse fraction was removed by centrifugation at 4° C at 2,000$\times g$ for 10 min. The supernatant was then centrifuged at 100,000$\times g$ for 60 min in a Spinco model L Ultracentrifuge, providing two

more fractions, particulate (sediment) and high speed supernatant. After protein precipitation of the various fractions the samples were passed through an ion exchange column (Dowex 50W X4). The column was washed with 40 ml. of glass-distilled water, and the ^{3}H-MA was then eluted with 1N HCl. Further details of the analytical procedure have been previously described (Stitzel & Lundborg, 1967).

In vitro experiments. Amine granules from cow adrenal medullae were prepared essentially as described by Hillarp (1958). The granules were resuspended in 0.3 M sucrose and stored at 0° C for use on the same or the next day. An aliquot (50 μl.) of the granule suspension, corresponding to about 125 μg of catecholamines, was transferred to 1 ml. of an incubation mixture (at 0° C) containing glycylglycine (0.31 M), ATP (0.0025 M) and $MgCl_2$ (0.0025 M). ^{3}H-MA was added in a concentration such that the total amount of labelled plus unlabelled drug was 3.0×10^{-4} M. Varying concentrations of reserpine or protriptyline were added to some of the incubation flasks. Incubations were performed without shaking at 0° or 31° C for 30 min.

Substances used. (±) ^{3}H-MA was prepared by the research laboratory of Hässle Ltd. in co-operation with this department (Hallhagen & Waldeck, to be published). Reserpine and protriptyline were generously supplied by Swedish Ciba Ltd. and Dr. C. A. Stone, of the Merck Institute for Therapeutic Research, respectively.

Data were calculated for significance using an analysis of variance.

RESULTS

Uptake of ^{3}H-MA by subcellular fractions of the mouse heart

Table 1 shows the subcellular distribution of ^{3}H-MA at three intervals after the injection of 40 μg/kg. The amount of labelled amine in the heart increased rapidly during the first 30 min and then declined slowly. The subcellular distribution indicates that the principal site of loss of ^{3}H-MA in the 24 hr period was from the supernatant fraction. A calculation of the amount of ^{3}H-MA in the particulate fraction as a percentage of the amount in the particulate plus supernatant fractions shows that a greater proportion of the amine is particle-bound at 24 hr than at 0.5 or 1 hr.

TABLE 1

EFFECT OF RESERPINE AND PROTRIPTYLINE ON THE SUBCELLULAR DISTRIBUTION OF ^{3}H-METARAMINOL (NG/G OF FRESH TISSUE) IN THE MOUSE HEART

The values given are means±S.E.M. Each determination was performed on six pooled hearts, and each figure is the mean of 4–6 determinations

Time after ^{3}H-MA (hr)	Treatment	Particulate	Supernatant	$\frac{P}{P+S}\times100$
½*	Control	8·45±0·85	37·68±1·83	18·3±1·1
	Reserpine	7·85±0·73	37·46±1·70	17·5±2·0
	Protriptyline	7·61±0·71	25·13±2·30$_\alpha$	23·3±1·3
1**	Control	6·40±0·86	29·76±3·46	18·8±1·2
	Reserpine	4·69±0·32†	24·36±1·21	16·1±0·6
	Protriptyline	6·15±0·80	16·64±1·53$_\alpha$	26·8±1·0
24**	Control	6·77±0·40	20·06±1·12	25·2±0·5
	Reserpine	2·79±0·34$_s$	18·02±1·76	13·9±1·0
	Protriptyline	5·76±0·33	16·32±1·02	26·2±0·6

* Animals were given reserpine or protriptyline 15 min before death.
** Animals were given reserpine or protriptyline 45 min before death.
† $P=0·05$. $_\alpha$ $P<0·01$. $_s$ $P<0·001$.

Effect of reserpine on the ^{3}H-MA content of the mouse heart

Reserpine administration 15 min before death caused no significant decrease in the ^{3}H-MA content of mouse heart fractions from animals examined 30 min after the injection of ^{3}H-MA (Table 1). Sixty minutes after receiving ^{3}H-MA (and 45 min after reserpine) there was a decrease in the amount of labelled amine in the particulate fraction ($P=0.05$). There was a slight tendency towards a lowering of the content in the supernatant fraction as well, but this was not significant ($P>0.10$). Animals which had been given ^{3}H-MA 24 hr previously showed a large decrease in the amount of tritiated amine recovered from the particulate fraction of the heart 45 min after a single intravenous injection of reserpine (Table 1).

Effect of protriptyline on the ^{3}H-MA content of the mouse heart

Groups of mice pretreated with ^{3}H-MA received an intravenous injection of protriptyline (10 mg/kg) 15 or 45 min before death.

Both 30 min (i.e., 15 min after protriptyline) and 60 min (i.e., 45 min after protriptyline) after ^{3}H-MA administration, protriptyline caused a loss of the labelled amine from the supernatant fraction ($P<0.001$). There was no significant decline in the particulate fractions (Table 1).

Protriptyline had much less effect on the content of ^{3}H-MA remaining in the heart when it was given 24 hr after the labelled amine.

Effect of reserpine and protriptyline on the in vitro *uptake of ^{3}H-MA by bovine adrenal medullary granules*

Reserpine, in concentrations from 10^{-8} to 10^{-6} M, caused only a slight impairment of the uptake of ^{3}H-MA by the granules of the adrenal medulla (Table 2). The highest concentration of reserpine (10^{-6} M) which reduced ^{3}H-MA uptake by only 25% caused an 80% blockade of uptake of ^{3}H-noradrenaline (Lundborg & Stitzel, 1967). Protriptyline, on the other hand, even in concentrations as high as 2×10^{-5} M, caused no inhibition of ^{3}H-MA uptake.

TABLE 2

INFLUENCE OF RESERPINE AND PROTRIPTYLINE ON THE UPTAKE OF ^{3}H-METARAMINOL BY BOVINE ADRENAL MEDULLARY GRANULES

The final concentration of metaraminol (labelled plus unlabelled) was 3×10^{-4}M. The percentage inhibition values are based upon 4–8 determinations

Compound	Concn. of compound (M)	Percent Inhibition
Reserpine	4×10^{-8}	5·9
Reserpine	4×10^{-7}	23·1
Reserpine	4×10^{-6}	25·7
Protriptyline	4×10^{-6}	0
Protriptyline	2×10^{-5}	0

DISCUSSION

It has been postulated that the mechanism for uptake of monoamines into adrenergic nerves consists of two major components: transport through the nerve cell membrane and an incorporation into the storage granule complex (Carlsson, Hillarp & Waldeck, 1963). Both of these mechanisms can be selectively blocked by drugs. Of the drugs

used in the present experiments protriptyline has been found to be a potent blocker at the nerve cell membrane, while reserpine inhibits the incorporation of monoamines into the adrenergic granules (Carlsson & Waldeck, 1965, 1966a). However, the existence of a reserpine-resistant uptake mechanism in the amine granules has also been pointed out (Lundborg, 1966 ; Lundborg & Stitzel, 1967).

^{3}H-MA has been used in our studies to examine the different mechanisms of drug-induced amine loss. This compound was chosen since it has been shown to use both the reserpine-resistant and the reserpine-sensitive mechanisms in adrenergic granules and also to utilize the concentrating mechanism located in the cell membrane. MA also has the additional advantage of not being a substrate for a number of enzyme systems, including monoamine oxidase and catechol-O-methyl-transferase (Gram & Wright, 1965).

Reserpine has little or no ability to reduce the amount of ^{3}H-MA which was retained in the heart during the first 30 min after the injection of the labelled compound. However, reserpine was able to deplete ^{3}H-MA at later time intervals, being particularly effective when given 24 hr after the intravenous administration of ^{3}H-MA. Carlsson & Waldeck (1966b) have reported similar findings for the whole heart. In our fractionation experiments, the primary site of action of reserpine was the particulate fraction. There appears to be a gradual transfer of particle-bound ^{3}H-MA from a reserpine-resistant to a reserpine-sensitive storage site during the 24 hr period following amine administration. This confirms the existence of a reserpine-resistant concentrating mechanism in adrenergic granules (Lundborg & Stitzel, 1967). Furthermore, Lundborg & Waldeck (1966) have shown that animals pretreated with reserpine accumlate ^{3}H-MA in approximately normal amounts during the first 30 min, but the retention at later time periods was impaired by reserpine.

It is difficult to state whether the reserpine-resistant and the reserpine-sensitive mechanisms represent two steps of amine accumulation in a single granule or whether they are present in separate populations of granules. It is interesting to note, however, that different pools of catecholamines have been observed in adrenal granules (Hillarp, 1960) and that Helle (1966) has recently isolated two separate but closely related soluble proteins from adrenal medullary granules.

Under normal conditions blockade of the membrane-pump by protriptyline apparently does not cause a depletion of noradrenaline (Carlsson & Waldeck, 1966a ; Malmfors, 1965), although as we and others have shown MA loss is accelerated. The relatively slow release induced by blockade of this uptake mechanism alone probably indicates that the concentration of free amine in the nerve cytoplasm is low and therefore appreciable leakage through the cell membrane does not occur. Carlsson (1966) has suggested that MA may be lost more rapidly than is noradrenaline because it is more lipid soluble and therefore can diffuse more readily across cell membranes.

In our experiments, protriptyline is much more effective in causing a loss of ^{3}H-MA from heart nerves at shorter intervals than when it is given 24 hr after injection of the labelled amine. The loss of amines which does occur at the 30 and 60 min time periods is almost exclusively from the supernatant fraction of the cell. These results support the view that protriptyline acts specifically at the level of the cell membrane rather than on the storage granules. Further support for such a hypothesis is found in our *in vitro*

data which demonstrate that, even in high concentrations, protriptyline does not inhibit the uptake of ^{3}H-MA by isolated adrenal granules. The fact that protriptyline is virtually ineffective at longer time intervals supports the view that there may be a gradual movement of ^{3}H-MA from a more to a less labile pool.

SUMMARY

1. Mice killed 30 min after the injection of ^{3}H-MA and 15 min after that of reserpine showed no significant decrease in ^{3}H-MA content in any subcellular fraction of the heart. Sixty minutes after receiving ^{3}H-MA there was a pronounced decrease in the amount of ^{3}H-MA in the particulate fraction of mice given reserpine 45 min before death.

2. The *in vitro* incorporation of ^{3}H-MA was slightly (25%) reduced by inclusion of reserpine in the incubation medium, while protriptyline had no effect on the uptake, suggesting that protriptyline probably interferes with amine accumulation at the cell membrane rather than in the storage granules.

3. Protriptyline was only partially effective in producing a loss of ^{3}H-MA when the labelled amine was given 24 hr previously. However, at shorter time intervals after ^{3}H-MA administration, protriptyline caused a more pronounced loss of ^{3}H-MA from the supernatant fraction of the mouse heart. There probably is a gradual movement of ^{3}H-MA from a more to a less accessible site.

4. It is tentatively concluded that particle-bound ^{3}H-MA is first taken up at a reserpine-resistant site and later is either transferred to or taken up in a separate, reserpine-sensitive, storage pool.

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