Journal of Neurochemistry, 1975. Vol. 25, pp. 915–917. Pergamon Press. Printed in Great Britain.

SHORT COMMUNICATION

Octopamine probably coexists with noradrenaline and serotonin in vesicles of pineal adrenergic nerves

(*Received* 13 *March* 1975. *Accepted* 19 *May* 1975)

THE PINEAL gland of the rat is densely innervated by sympathetic fibres which control the metabolism of pineal parenchymal cells through the liberation of noradrenaline (AXELROD, 1974; KLEIN, 1974). However, these nerves are unique because they also contain serotonin (PELLEGRINO DE IRALDI *et al.*, 1963; OWMAN, 1964; NEFF *et al.*, 1969). This indoleamine, synthesized by the pinealocytes is taken up by the nerves and stored together with noradrenaline (JAIM-ETCHEVERRY & ZIEHER, 1971, 1974). The possible coexistence of both amines within storage vesicles is suggested by cytochemical observations at the ultrastructural level (JAIM-ETCHEVERRY & ZIEHER, 1968) and by results from biochemical and pharmacologic experiments (JAIM-ETCHEVERRY & ZIEHER, 1973, 1974). For example, when neuronal serotonin is reduced either by drugs or by stimulation of the postsynaptic β-adrenergic receptor in the pineal, there is a marked and selective rise in pineal noradrenaline (JAIM-ETCHEVERRY & ZIEHER, 1971, 1975). On the contrary, the depletion of noradrenaline increases neuronal serotonin (ZWEIG & AXELROD, 1969).

Octopamine has been recently identified as a naturally occurring amine in mammalian adrenergic nerves and the characteristics of the mechanisms responsible for its synthesis, storage and release, led to the suggestion that octopamine may serve as a 'cotransmitter', a similar role to that proposed for pineal serotonin (MOLINOFF & AXELROD, 1969, 1972; MOLINOFF *et al.*, 1969). It is therefore possible that octopamine, which is present at a relatively high concentration in the rat pineal gland (MOLINOFF & AXELROD, 1972; SAAVEDRA, 1974), may also coexist with noradrenaline and serotonin in pineal nerve vesicles. If this assumption is correct, octopamine levels should be selectively modified in the pineal in response to changes in the intravesicular concentration of one of the other amines. To explore this possibility, we depleted pharmacologically the serotonin stored in pineal nerves and studied the changes in the octopamine content of the pineal and salivary glands.

To reduce neuronal serotonin we used two drugs which act through different mechanisms: *p*-chlorophenylalanine (PCP) and desmethylimipramine (DMI). The marked reduction of pineal serotonin produced by PCP at the dose and time interval used, was originally considered to reflect the inhibition of pineal tryptophan hydroxylase (tryptophan 5-monooxygenase EC 1.14.16.4) as in the brain (KOE & WEISSMAN, 1966; JAIM-ETCHEVERRY & ZIEHER, 1974) but recent data indicate that the pineal enzyme has a differential sensitivity to PCP (DEGUCHI & BARCHAS, 1972). On the other hand, DMI blocks the uptake of serotonin in pineal nerves thereby reducing its intraneuronal concentration without modifying either serotonin turnover or its total content in the gland (NEFF *et al.*, 1969). We have previously reported that in both experimental conditions there is a selective increase in pineal noradrenaline (JAIM-ETCHEVERRY & ZIEHER, 1971).

Adult Wistar rats of both sexes (150–200 g) were maintained for 2 weeks before the experiments under diurnal lighting (lights on at 7.00 a.m. and off at 7.00 p.m.) and injected intraperitoneally 48 and 24 h before killing with one of the following drugs: *p*-chlorophenylalanine methyl ester (H69/17, Labkemi, Goteborg, Sweden) 300 mg/kg; desmethylimipramine HCl (Pertrofran, Ciba-Geigy, Buenos Aires, Argentina) 20 mg/kg and 10 mg/kg and nialamide HCl (Niamid, Pfizer, Buenos Aires, Argentina) 100 mg/kg. Drugs were dissolved in saline and control rats received saline at the same intervals. The animals were killed by decapitation 24 h after the last injection, between 2.00 and 3.00 p.m. In a group of animals, the pineal was surgically denervated by the bilateral excision of the superior cervical ganglia. Some of these rats were injected 2 weeks later with PCP while the rest served as controls. The concentration of octopamine was determined in extracts of pineal and salivary glands with the procedure described by MOLINOFF *et al.* (1969) in which octopamine is methylated by phenylethanolamine *N*-methyltransferase (EC 2.1.1.X) in the presence of labelled *S*-adenosyl-methionine which acts as a methyl donor. The radioactive products of the methylation of octopamine, synephrine and dimethyloctopamine were identified by thin layer chromatography (MOLINOFF *et al.*, 1969).

Table 1 shows that a relatively high concentration of octopamine was found in the pineal gland of the normal rat, confirming previous reports (MOLINOFF & AXELROD, 1972; SAAVEDRA, 1974). Following administration of PCP or DMI, there was a marked rise in pineal octopamine content while amine levels in the salivary gland were unchanged. Inhibition of monoamine oxidase (monoamine: O_2 oxidoreductase (deaminating) EC 1.4.3.4.; MAO) by nialamide, increased octopamine in both pineal and salivary glands. The chromatographic profiles shown in Fig. 1 indicate that the products formed during the assay of normal pineal extracts, resulted from the methylation of authentic octopamine because almost all the radioactivity was isographic with methylated octopamine derivatives. Moreover, it was demonstrated that the amine was stored in sympathetic nerves because it became barely detectable following pineal denervation. Also shown in the figure are the patterns of extracts from pineals of rats treated with PCP and DMI, indicating that the increase described was due to an elevation of the authentic amine and that it occurred in adrenergic nerves because surgical denervation of the gland abolished the increase in octopamine produced by PCP injection.

Abbreviations used: PCP, *p*-chlorophenylalanine; DMI, desmethylimipramine; MAO, monoamine oxidase.

TABLE 1. CONTENT OF OCTOPAMINE IN THE PINEAL AND SALIVARY GLANDS AFTER DIFFERENT TREATMENTS

Treatment	Pineal gland (pg per pineal)	(%)	Salivary gland (ng per g)	(%)
Saline	309 ± 26	100	193·47 ± 12·51	100
p-chlorophenylalanine	1558 ± 105*	503	219·93 ± 22·94	113
Desmethylimipramine	1245 ± 117*	402	191·99 ± 12·61	99
Nialamide	1681 ± 171*	522	665·56 ± 23·72*	344

Dosages and time schedule of administration of drugs are given in the text.

Results represent the mean ± S.E.M. for 6–8 groups, each of 12–16 rats.

* Significance of differences as compared to control: $P < 0.001$.

Octopamine was assayed with the method of MOLINOFF *et al.* (1969). Extracts from pineal and salivary glands were made by homogenisation in ice-cold 0·1 M-Tris–HCl buffer pH 8,6, containing pargyline (50 μg/ml) to inhibit monoamine oxidase. The homogenate was heated at 95°C for 5 min and after centrifugation, 500 μl of the supernatant were placed in glass-stoppered tubes. The reaction was initiated by the addition of 1 nmol of [^{14}C]S-adenosyl-l-methionine (sp. act. 52 mCi/mM; New England Nuclear Corp.) and 10 μl of a partially purified preparation of phenylethanolamine-*N*-methyltransferase obtained from bovine adrenal medulla. Blanks were obtained by incubating 500 μl of the buffer and internal standards of 20 ng of DL-octopamine HCl were added to a duplicate of each experimental sample. The reaction was stopped by addition of 0·5 ml of 0·5 M-borate buffer pH 10 and the radioactive products formed were extracted in a mixture of toluene:isoamyl alcohol (3:2). After evaporation of the organic phase, the radioactivity was counted.

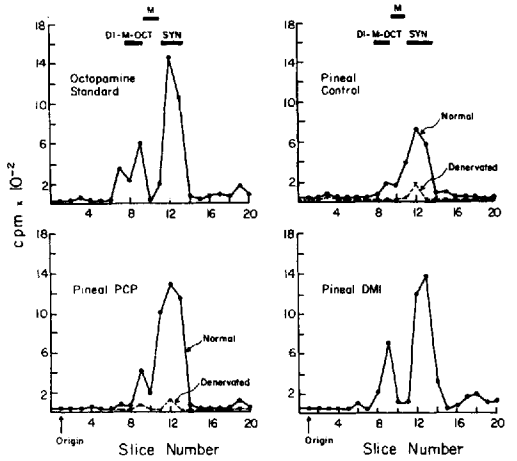

FIG. 1. Identification of the radioactive products formed in the assay of octopamine. Standards of DL-octopamine HCl and extracts of pineal glands from normal, denervated and treated rats were carried through the sequence described in Table 1. Following evaporation of the organic phase, the residue was taken up in ethanol to which non-radioactive standards metanephrine (M), synephrine (SYN) and dimethyloctopamine (DI-M-OCT) were added and spotted on precoated silica gel sheets (Eastman Chromagram). After development in *n*-buthanol saturated with 1 N-HCl, the sheets were stained with diazotized *p*-nitroaniline, cut into slices (0·6 × 1·5 cm) and transferred to counting vials containing 1 ml ethanol. The radioactivity was determined after addition of 10 ml of phosphor to each vial. Chromatographic profiles shown are: (a), octopamine standard; (b), control pineal, normal and surgically denervated; (c), normal and surgically denervated pineal of rats treated with *p*-chlorophenylalanine 300 mg/kg 48 and 24 h before killing; and (d), pineal gland of rats treated with desmethylimipramine 20 and 10 mg/kg 48 and 24 h before killing.

The interpretation of the effects of PCP on biogenic amine metabolism is difficult because the drug does not only inhibit tryptophan hydroxylase but also decreases the activity of other enzymes related to the synthesis of catecholamines (KOE & WEISSMAN, 1966) and may also modify tyrosine metabolism. This is the reason why the levels of octopamine in the pineal were also studied after administration of DMI which reduces the content of neuronal serotonin like PCP but through a completely different mechanism. A similar approach was previously used to demonstrate that the depletion of serotonin from pineal nerves determines a selective rise of their noradrenaline content (JAIM-ETCHEVERRY & ZIEHER, 1971). As the results of the present experiments have shown, the administration of PCP and DMI produced a marked increase in pineal octopamine. Such an effect could result from a direct and general action of the drugs on the synthesis or degradation of the amine in the nerves, as has been postulated to explain the increase of brain octopamine following PCP (SAAVEDRA *et al.*, 1974), or from their conversion to products capable of being enzymatically methylated in the assay reaction. In both cases, octopamine levels should also rise in other sympathetically innervated organs. The results show that in the salivary gland, receiving its innervation from the superior cervical ganglion as the pineal, the content of octopamine was unchanged after PCP or DMI treatment but was increased by MAO inhibition which blocks non-selectively octopamine degradation (KAKIMOTO & ARMSTRONG, 1962; KOPIN *et al.*, 1964). However, the interpretation of the results obtained when studying octopamine metabolism in the salivary gland should take into account recent data showing that 80% of the octopamine found in the gland is not localized in its adrenergic nerves but is stored in other compartments, probably octopaminergic cells (COYLE *et al.*, 1974). Therefore, the failure to increase octopamine in the salivary gland after PCP or DMI treatments may reflect differences in the metabolism of the amine in the glandular tissue and not in the physiology of adrenergic nerves. In this case, octopamine should increase in the nerves of the salivary gland as in those of the pineal but this change would not be reflected in the total octopamine content of the gland. Assuming that 20% of the octopamine present in the salivary gland is in adrenergic nerves and that following PCP or DMI the amine increases in this neuronal compartment as it does in the pineal, a significant increase of about 100% in total salivary gland octopamine should be observed. This was not the case since the content of octopamine in the salivary gland was not modified by PCP or DMI. Therefore, since the increase of pineal octopamine seems to be selective, it must be related to some characteristic of pineal nerves not shared with other postganglionic sympathetic terminals. The most evident of these characteristics and the one which is modified by both experimental procedures used, is the presence of serotonin in the fibres innervating the pineal.

Therefore, the most likely interpretation of the increase in pineal octopamine is similar to that previously advanced

for explaining the rise of noradrenaline following depletion of neuronal serotonin in the pineal, i.e. the increased availability of storage sites within adrenergic nerve vesicles (JAIM-ETCHEVERRY & ZIEHER, 1971). These vesicles, once depleted partially of their content, could store more octopamine and protect it from destruction by MAO; this leading to an elevation of octopamine levels. The importance of MAO activity in the metabolism of octopamine has been shown by several studies (MOLINOFF *et al.*, 1969; KAKIMOTO & ARMSTRONG, 1962; KOPIN *et al.*, 1964) and is also demonstrated by the increase in pineal octopamine after MAO inhibition. The results suggest that when neuronal serotonin is depleted, the storage sites which become available in nerve vesicles are sufficient to protect from MAO, almost all the octopamine in pineal nerves because in this condition its concentration is similar to that found when MAO is inhibited. However, the interpretation of the changes produced by MAO inhibition on the dynamics of intravesicular storage of amines in pineal nerves may be more complex and is being currently investigated. Results of recent experiments suggest that following MAO inhibition, although serotonin remains in pineal nerves, its concentration is lowered within nerve vesicles (unpublished observations). This probably results from the displacement of intravesicular serotonin by the noradrenaline and octopamine which accumulate and gain access to the vesicles. Thus, the inhibition of MAO in pineal nerves would have a similar effect to that of the depletion of serotonin from these nerves.

Since octopamine is synthesized, accumulated, stored and released by sympathetic nerves (KAKIMOTO & ARMSTRONG, 1962; KOPIN *et al.*, 1964; SNYDER *et al.*, 1964; KOPIN *et al.*, 1965; MUSACCHIO *et al.*, 1965; MOLINOFF & AXELROD, 1969; MOLINOFF *et al.*, 1969; MOLINOFF & AXELROD, 1972), it may act on postsynaptic structures either by modifying the amount of neurotransmitter released, by interfering with its effects on the receptors or by directly interacting with them. Such an interaction has been shown not only in non-mammalian species (LEVITAN & BARONDES, 1974) but also in the pineal gland of the rat where octopamine mimicks the effects of noradrenaline although producing a less potent response (KLEIN & WELLER, 1973). Moreover, variations in octopamine levels similar to those reported here as a result of drug-induced changes in the storage capacity of nerve vesicles, may be of relevance for the neural control of pineal function. This seems possible because the availability of storage sites in pineal adrenergic vesicles is modified physiologically, for example when serotonin decreases in the gland at the onset of darkness.

The adrenergic nerve vesicles of the rat pineal gland, either as a result of the non-specificity of the amine synthesis in the neuron (e.g. octopamine) or of the uptake in the terminal portions of the cell (e.g. serotonin), are apparently capable of storing several amines which may serve as 'cotransmitters' and which might modify neurotransmission. Such properties may constitute a general feature of monoaminergic nerve vesicles.

Acknowledgements—We thank Dr. J. M. SAAVEDRA for discussing aspects of the octopamine assay and Drs. D. C. KLEIN and S. Z. LANGER for many helpful comments. For kind gift of drugs we are grateful to Abbot Laboratories, Ciba-Geigy and Pfizer, Argentina and to Dr. F. C. NACHOD from the Sterling Winthrop Research Institute, Rensselaer, N.Y., U.S.A. This work was supported by a grant from the Consejo Nacional de Investigaciones Cientificas y Técnicas, Argentina (6050a/74) and the National Institutes of Health, U.S.A. (5 RO1 NS 06953-08 NEUA).

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