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Short Communication

Tyrosine Hydroxylase Activity Increases in Pineal Sympathetic Nerves after Depletion of Neuronal Serotonin

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Summary. The injection of p-chlorophenylalanine (PCPA) acutely reduced serotonin in the pineal gland of the rat and selectively elevated the noradrenaline (NA) content during the subsequent 24 h. The activity of tyrosine hydroxylase (TH) also increased in intact glands during the first 6 h after PCPA injection but returned to normal at 24 h. This enhancement of enzyme activity was only observed in the presence of a non-saturating concentration of the cofactor. Serotonin depletion by PCPA cannot directly account for the increased enzyme activity, because the amine does not modify TH activity. Moreover, this increase is restricted to the pineal, since in other sympathetically innervated organs, such as the atria, PCPA produced an acute but transient reduction in TH activity. The elevation described here is not due to a net increase in the amount of enzymatic protein, because TH activity is similar in pineal homogenates from treated and control rats when a saturating concentration of the cofactor was used. The availability of storage sites in pineal nerve vesicles due to serotonin depletion, seems to release TH activity from the negative control exerted by cytoplasmic catecholamines. Enzyme activity in the pineal is acutely enhanced until a new steady state is reached at a higher concentration of endogenous NA.

Key words: Tyrosine hydroxylase – Noradrenaline – Serotonin – Pineal gland – Amine storage.

INTRODUCTION

The pineal gland of the rat is innervated by sympathetic fibers that contain the physiologic neurotransmitter, noradrenaline (NA) (see Axelrod, 1974). Due to the relative unspecificity of the mechanisms of amine uptake and synthesis, other amines such as serotonin and octopamine are also present in these nerves. There is morphological, biochemical and pharmacological evidence suggesting that these amines coexist in the synaptic vesicles characteristic of sympathetic nerves (Jaim-Etcheverry and Zieher, 1971, 1975a, b). In support of this mechanism of storage, it has been shown that when one of the amines is depleted from pineal nerves, the concentration of the other increases (Zweig and Axelrod, 1969; Jaim-Etcheverry and Zieher, 1971). Moreover, this elevation is selective for the pineal, i.e., NA levels are not modified in other sympathetically innervated organs.

To explain these findings we have proposed that the vacancy of vesicular storage sites produced by serotonin depletion, most probably results in the entrance of extravesicular catecholamines within partially emptied vesicles. It is known that catecholamines exert a negative control on their own synthesis by a competitive antagonism with the pteridine cofactor of tyrosine hydroxylase (TH), the enzyme responsible for their formation (Nagatsu et al., 1964; Udenfriend et al., 1965; Rubio, 1976; see Weiner, 1970). Therefore, the reduction of the small pool of extravesicular catecholamines by the uptake of the amines into the vesicles could enhance TH activity (Spector et al., 1967; see Weiner et al., 1972) and thus explain the increase in pineal NA observed after a partial depletion of vesicular content of serotonin.

To investigate this possibility, we studied the activity of TH at different time intervals after depletion of serotonin from pineal adrenergic nerves. For this purpose we injected p-chlorophenylalanine (PCPA), since this drug effectively decreases pineal serotonin by interfering with the activity of the enzyme responsible for its synthesis (Koe and Weissman, 1966; Deguchi and Barchas, 1972).

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MATERIAL AND METHODS

Female Wistar rats of 150–200 g used in this study were kept under diurnal lighting with the lights on from 07.00 h to 19.00 h for at least 2 weeks before the experiments. In some rats, p-chlorophenylalanine methyl ester (PCPA) dissolved in saline was injected (300 mg/kg intraperitoneally) at different times before killing the animals. All animals were decapitated at 13.30 h in order to avoid diurnal variations in amine content. Rats injected at the same times with saline served as controls.

Tyrosine hydroxylase activity was assayed in pineal glands of control rats and in those of animals which received PCPA 2, 4, 6 and 24 h before death. Enzyme activity was assayed both in homogenates and in intact tissues. For the former procedure, 5 pineals were homogenized in the cold in 200 µl of 150 mM KCl; 100 µl of this homogenate were used as the source of the enzyme. TH activity was determined with the coupled decarboxylation procedure of Waymire et al. (1971) after an incubation of 30 min at 37° C. The concentration of the cofactor, 2 amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine hydrochloride ($DMPH_4$) was either non-saturating (0.1 mM) or saturating (1 mM) as described in Results. When the influence of serotonin on TH activity was studied, pineal homogenates were preincubated at 37° C in the medium without labeled tyrosine for 10 min in the presence of 10^{-3} M serotonin. At the end of this period, ^{14}C-tyrosine was added and the incubation continued for 30 min. The activity of the enzyme was also assayed in intact tissue. For this procedure, 5 pineals or a single atrium were incubated during 20 min in the assay medium containing the radioactive substrate and the cofactor (Rubio, 1976). Blanks were obtained in the presence of 2 mM 3-iodo-tyrosine. The radioactivity of the CO_2 formed in the reaction was counted by liquid scintillation spectrometry with an efficiency of 83%.

The amine content of the pineal glands was determined by extraction of amines from the glands following incubation for TH assay with 0.4 N perchloric acid containing $Na_2S_2O_5$ and EDTA (Atack, 1973). Noradrenaline and serotonin were separated from the supernatant of the centrifuged homogenate by cation exchange column chromatography as described by Atack and Magnusson (1970). Mean recoveries were 93% for NA and 72% for serotonin; values obtained were not corrected for recovery. Noradrenaline and serotonin concentrations were determined in the same extract in the corresponding eluates according to the techniques described by Häggendal (1963) and Magnusson (1973), respectively. In the case of the atria, only the NA content was determined.

For each group 5–6 experiments were performed and results were expressed as mean values ± S.E.M. The significance of differences was analyzed by Student's *t*-test.

Drugs used were: p-chlorophenylalanine methyl ester (H69/17, Labkemi, Sweden); 2 amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine hydrochloride and serotonin creatinine sulphate monohydrate (Aldrich Chemical Co., U.S.A.); 3-iodo-tyrosine (Sigma Chemical Co., U.S.A.) and 1-^{14}C-L-tyrosine (52 µCi/µmol, New England Nuclear Corp., U.S.A.).

RESULTS AND DISCUSSION

Pharmacological procedures that deplete serotonin from the adrenergic nerves of rat pineal gland through different mechanisms cause a marked increase in the NA contained in these nerves (Jaim-Etcheverry and Zieher, 1971, 1975a, b). In the present experiments, the content of serotonin was reduced in the pineal by the injection of PCPA. This compound, an inhibitor of the activity of the enzyme that converts tryptophan to 5-OH-tryptophan (Koe and Weissman, 1966), seems to deplete pineal serotonin by lowering the affinity of tryptophan hydroxylase for its substrate (Deguchi and Barchas, 1972). Figure 1a shows the marked changes in the content of serotonin and of NA in the pineal gland that occur during the 24 h after the injection of PCPA. Serotonin was depleted by 80% already 2 h after injection of PCPA, and this reduction was maintained during the following 22 h. On the other hand, the NA content was markedly elevated after PCPA treatment. Two hours after injection, NA levels were somewhat increased and this elevation continued at 4, 6 and 24 h. The analysis of the time course of NA changes in the pineal indicates that the elevation was more rapid during the initial 6 h after the injection of PCPA than in the subsequent period. Thus, during the first 4 h after PCPA, pineal NA rose 52%, a further increase of 16% took place in the following 2 h and, in the subsequent 18 h, NA content was elevated by 24%.

The increase in pineal NA observed after PCPA is selective, i.e., limited to this organ and not found in other sympathetically innervated tissue. Figure 1a also shows that the content of NA in the atria was practically unchanged during the 24 h period after PCPA injection. This finding confirms previous observations in several sympathetically innervated organs after the administration of PCPA and of other compounds that deplete pineal serotonin and increase NA levels in the gland (Jaim-Etcheverry and Zieher, 1971, 1975b).

To explain this selective increase of pineal NA, we have advanced the hypothesis that both serotonin and catecholamines coexist in the adrenergic vesicles and that serotonin depletion from these vesicles, as shown by electron microscopic cytochemistry, leaves available storage space that is occupied by catecholamines. Initially, cytoplasmic catecholamines would enter into the partially emptied vesicles, thus escaping from enzymatic deamination. This would release TH from the inhibition exerted by cytoplasmic NA, dopamine and other catechol derivatives (see Introduction) and the synthesis of NA would be enhanced immediately after serotonin depletion until a new steady state is reached in which NA levels are higher than normal. Since such a mechanism would result from the interplay of regulatory processes taking place within the nerve terminal, to investigate changes in TH activity, it would be necessary to maintain the structural and biochemical organization of the ending. Therefore, enzymatic activity was measured in intact pineals and in the presence of a cofactor concentration in the incubation medium similar to the estimated intraneuronal concentration of the

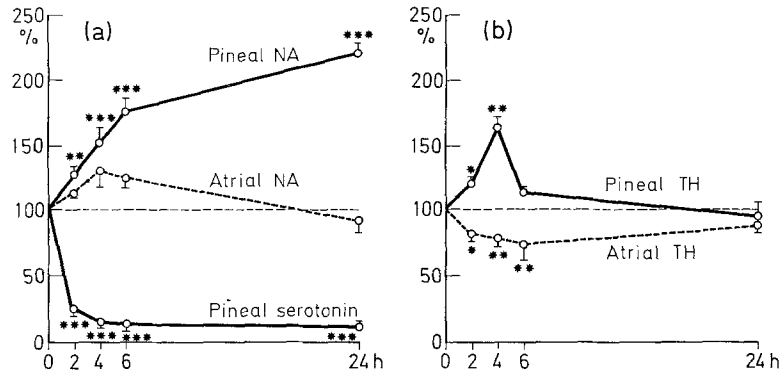

Fig. 1
(a) Changes in the content of noradrenaline (NA) in the pineal gland and in the atria and of serotonin in the pineal of rats injected at different time intervals with PCPA. Absolute values for controls were: pineal NA 14.47 ± 0.91 pmoles/pineal; pineal serotonin 318.19 ± 11.91 pmoles/pineal; atrial NA 7.41 ± 0.78 nmoles/g.
(b) Changes in the activity of tyrosine hydroxylase (TH) in the presence of 0.1 mM $DMPH_4$ in intact pineals from rats injected at different time intervals with PCPA. Absolute values for TH activity were: pineal 309 ± 20 dpm Dopa formed/pineal/20 min; atria 8.76 ± 2.12×10^3 dpm Dopa formed/g/20 min. These are mean values ± S.E.M. of 5–6 experiments for each time interval. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared with control values

natural cofactor (0.1 mM $DMPH_4$) (Weiner et al., 1972). Figure 1 b shows that under these conditions, TH activity was increased 2 h after PCPA and was markedly elevated at 4 h. Although at 6 h enzymatic activity was elevated in comparison to controls, it was already reduced with respect to the activity found at 4 h. At 24 h, TH activity was again normal. As is apparent from this description, the time course of the elevation of TH activity roughly parallels the rate of increase in endogenous NA.

To investigate whether the mechanism involved was competitive with the cofactor, the activity of TH in intact glands was determined in the presence of a saturating concentration of $DMPH_4$ (1 mM) (Nagatsu et al., 1964). With excess cofactor, TH activity (expressed as dpm Dopa formed/pineal/20 min) was 420 ± 18 in control rats and 433 ± 21 in the glands of animals that received PCPA 4 h before. As expected, when a saturating concentration of cofactor was used, enzymatic activity was higher than in the presence of a lower cofactor concentration.

The increase of TH activity does not seem to result from an increase in the amount of enzymatic protein. In the presence of a saturating concentration of the cofactor, TH activity (expressed as pmol Dopa formed/pineal/h) was 15.8 ± 1.2 in homogenates from normal pineal glands and 16.8 ± 2.0 in those obtained from rats treated with PCPA 4 h earlier.

These results suggest that, acutely after PCPA injection, TH is released from some negative control mechanism which is competitive with the cofactor. The intraneuronal concentration of serotonin could have such a role and its depletion by PCPA could enhance TH activity. To test this possibility, a high concentration of serotonin was added to the incubation medium in which TH activity was assayed in the presence of 0.1 mM $DMPH_4$. Under these conditions, TH activity in homogenates of control pineals (expressed as pmol Dopa formed/pineal/h) was 6.8 ± 0.8, while in the presence of 1 mM serotonin it was 7.0 ± 0.4. If serotonin would have been involved in the control of TH activity, the latter should have been reduced by the addition of the amine to the incubation medium. Moreover, this lack of effect of serotonin on TH activity is in accordance with studies on the structure-activity relationships of indole derivatives as TH inhibitors (Zhelyaskov et al., 1968).

The increase in TH activity could also result from a direct effect of PCPA on enzymatic activity in peripheral adrenergic nerves. To investigate this possibility, TH activity was determined in intact atria from normal and PCPA-treated rats. As shown in Figure 1 b, TH activity was not increased at any time after PCPA injection. On the contrary, the compound seems to exert a direct and acute inhibitory effect on TH activity. This inhibition is transient and TH activity in atria returns to normal 24 h after PCPA, a finding consistent with previous reports (Koe and Weissman, 1966). However, most probably the real increase of TH activity in the pineal produced by the disappearance of serotonin from nerve vesicles is greater than the values reported, since they result from opposite effects of the compound on enzyme activity.

The results indicate that the partial depletion of the amines contained within synaptic vesicles of peripheral adrenergic nerves enhances NA synthesis until the depleted stores are replenished and a new equilibrium is reached. In the case of the pineal, this interpretation is in line with physiological and pharmacological observations on the nature of the control exerted by adrenergic nerves on pineal indole metabolism related to the light-dark cycle (for discussion see Axelrod, 1974; Klein, 1974; Brownstein, 1975). At the onset of darkness, the activity of the nerves which supply the pineal increases. The released neurotransmitter acts on supersensitive β-adrenergic receptors

on pinealocytes and enhances the conversion of serotonin to its acetylated derivatives. This in turn decreases serotonin levels in the gland and, as suggested by pharmacological experiments in which this situation was mimicked (Jaim-Etcheverry and Zieher, 1975b), also in the adrenergic nerves. The partial vacancy of storage sites in the terminals would lead to an increase of TH activity and of pineal NA during the dark part of the cycle. Both phenomena have been reported to occur in the pineal at night (Brownstein and Axelrod, 1974; McGeer and McGeer, 1966). The enhanced release of NA would contribute to the development of receptor subsensitivity during the night. As a result, the serotonin concentration builds up in the pinealocytes and the amine is incorporated into the nerves. During the day, the presence of serotonin in adrenergic vesicles most probably serves to diminish the amount of NA released from each vesicle by the nerve impulse and thus the amine may play a modulatory role of neurotransmission in the pineal.

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