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ON THE PHYSIOLOGICAL ROLE OF NEUROCUPREIN: APONEUROCUPREIN IS AN INHIBITOR OF DOPAMINE β-MONOOXYGENASE

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SUMMARY: The apoform of neurocuprein, the copper protein from brain and chromaffin granules, was found to be a potent inhibitor of the hydroxylating activity of dopamine β-monooxygenase, whereas the holoform of neurocuprein has no effect on the activity of the enzyme. The inhibiting capacity of neurocuprein may be due to the property of the apoprotein to chelate copper from the enzyme. A role of neurocuprein as an endogenous protein regulator of dopamine β-monooxygenase is suggested.

Brain and adrenal medulla are known to contain a monocopper protein, named neurocuprein or EACP (extremely acidic copper protein) which is the main soluble copper-containing protein of these tissues (1-4). The physiological function of neurocuprein is unknown yet. Thus, no enzymatic activities were found for this protein.Besides, properties of neurocuprein differ significantly from those of metallothioneins,acidic thiol-rich proteins participating in accumulation, storage and transfer of some heavy metals, including copper (5,6). In order to elucidate the role of neurocuprein it was necessary to study interrelationships between this protein and copper-containing enzymes of brain and adrenal medulla.

In this communication we present results concerning the interaction of neurocuprein with the medullar copper-containing enzyme, dopamine β-monooxygenase,which is known to have, besides its hydroxylating, some o-diphenol oxidase activity (7). The data obtained show the possible function of neurocuprein as a factor regulating hydroxylating activity of dopamine β-monooxygenase.

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MATERIALS AND METHODS

Chromaffin granules were isolated from bovine adrenal medulla as described by Hoffman et al. (8). The granules obtained were lysed by freezing-thawing and the membrane fraction was removed by centrifugation. The supernatant was dialysed against 10 mM sodium acetate, pH 6.2, containing 100 mM sodium chloride, for 48 h. The solution was used as a source of soluble forms of dopamine β-monooxygenase and EACP (medullar neurocuprein).

Dopamine β-monooxygenase was prepared essentially according to Saxena and Fleming (9). The dialysed solution was applied to a DEAE-cellulose column (3x10 cm), equilibrated with 10 mM sodium acetate, pH 6.2. Active fractions were eluted with 100 mM sodium chloride, concentrated on an Amicon PM-30 membrane and passed through a Sepharose CL-6B column (1.5x80 cm), equilibrated with 50 mM HEPES buffer, pH 7.4 (or 50 mM sodium acetate, pH 6.2) containing 200 mM sodium chloride. After elution of the enzyme neurocuprein was eluted with 100 mM sodium acetate, pH 6.0, containing 500 mM sodium chloride and further was purified according to Grigoryan et al. (2). Neurocuprein from bovine brain was obtained as described in (1). Neurocupreins from brain and medulla used in this work were electrophoretically homogeneous. Their antigenic similarity was manifested by the immunodiffusion assay.

The copper-free form of neurocuprein was prepared by the treatment of the protein with diethyldithiocarbamate. The excess of the chelator was removed using DEAE-cellulose. Copper in preparations was assayed chemically with tetraethylthiuram disulphide (10).

The protein concentration was determined according to Lowry et al. (11). The concentration of dopamine β-monooxygenase was estimated from absorption coefficient, $E_{280}^{1\%}$, of 12.4 (12).

The hydroxylating activity of dopamine β-monooxygenase was assayed at 25°C, using tyramine as a substrate and ascorbate as a cosubstrate. The oxygen uptake was monitored as described by Blackburn et al. (13) on a Beckman M-0260 oxygen analyzer equiped with a Clark-type oxygen electrode and an Omniscribe recorder, B-5000. The preparations obtained had the specific activity of 20 μmoles of oxygen consumed by 1 mg of protein per min. The oxidase activity of dopamine β-monooxygenase was followed as in (7) using catechol and dopamine as substrates. No cosubstrate is necessary in this case.

RESULTS

It was found that neurocuprein from bovine brain and chromaffin granules has no effect on the oxygen uptake by the reaction mixture containing ascorbate, tyramine and dopamine β-monooxygenase. However, when the apoform of neurocuprein was added to the mixture the effective inhibition of the monooxygenase activity was observed (Fig. 1). The inhibition was found to depend on the concentration of aponeurocuprein (Fig. 2). These results indicate the high effectivity of aponeurocuprein as an inhibitor of dopamine β-monooxygenase. Thus, 50% inhibition of the enzyme was achieved at the

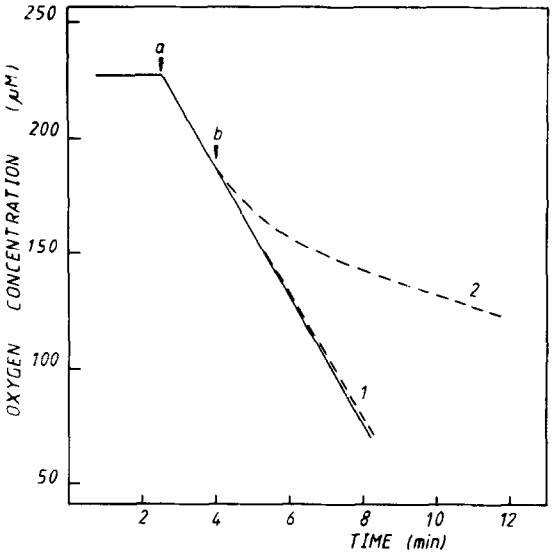

Fig. 1. The effect of neurocuprein and aponeurocuprein on tyramine-hydroxylase activity of dopamine β-monooxygenase. The reaction mixture contained 200 mM sodium acetate, pH 5.0, 20 μmoles of tyramine, 10 μmoles of ascorbate and 0.5 μmoles of catalase. Kinetics of the oxygen uptake were obtained: (——) - in the presence of 5 nmoles of dopamine β-monooxygenase and (- - -) - after the addition of 2.4 μmoles of neurocuprein (1) or aponeurocuprein (2). Arrows indicate the additions of the enzyme (a) and neurocuprein or aponeurocuprein (b) to the reaction mixture.

100-fold molar excess of aponeurocuprein. According to these data aponeurocuprein as an inhibitor of dopamine β-monooxygenase is as potent as diethyldithiocarbamate or cyanide, which are known to

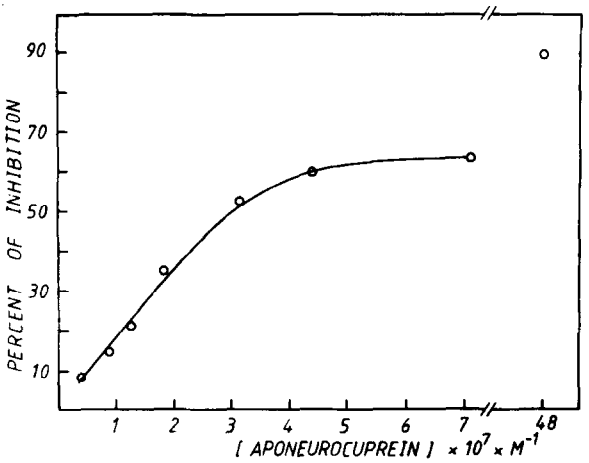

Fig. 2. The dependence of the inhibition of dopamine β-monooxygenase on concentration of aponeurocuprein. The reaction conditions were the same as described in the legend to Fig. 1.

be the two most powerful inhibitors of the enzyme (14), and two orders of magnitude more effective than EDTA.

The first, simplest and the most plausible assumption on the mechanism of the inhibition of dopamine β-monooxygenase by aponeurocuprein is the interaction of aponeurocuprein with the enzyme copper. Several lines of evidences were obtained supporting this possibility. First of all, the preincubation of aponeurocuprein with stoichiometric amounts of copper resulted progressively in the reconstitution of the protein (the holoform formation) with concomitant elimination of the inhibiting capacity of the reconstituted protein (Fig. 3). Further, when dopamine β-monooxygenase was incubated with aponeurocuprein for 1-2 h and then the mixture was gel-filtrated through Sephadex G-50 we have observed that at least a part of copper was transfered from the enzyme to the pro-

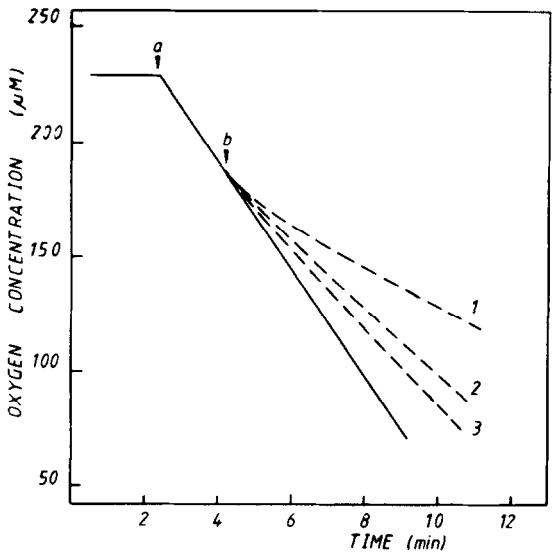

Fig. 3. The elimination of the inhibiting capacity of aponeurocuprein after its preincubation with Cu^{+2}. The reaction conditions were the same as described in the legend to Fig. 1 besides concentrations of dopamine β-monooxygenase and aponeurocuprein were 4 nmoles and 0.6 μmoles, respectively. (a) - the enzyme was added (——), (b) - aponeurocupreins were added: 1 - without preincubation with Cu^{+2}, 2 - after preincubation with stoichiometric amount of Cu^{+2} for 30 min, 3 - after preincubation with stoichiometric amount of Cu^{+2} for 60 min.

tein. This result shows clearly the chelating property of aponeurocuprein. In this connection it should be mentioned that the copper of dopamine β-monooxygenase (so-called type 2 copper) is known to be characterized by its accessibility to water. Hence, the activity of the enzyme should be very sensitive to chelating agents. Indeed, many chelators of copper are inhibitors of dopamine β-monooxygenase (14-17).

Further, we observed that when aponeurocuprein was added to the reaction mixture to assay the oxidase activity of dopamine β-monooxygenase no inhibition was noted with catechol as a substrate and only a weak inhibition was noted with dopamine as a substrate (Fig. 4). Thus, the oxidase activity of dopamine β-monooxygenase was practically unaffected by aponeurocuprein, whereas the hydroxylating function of the enzyme suffers under effect of aponeurocuprein.

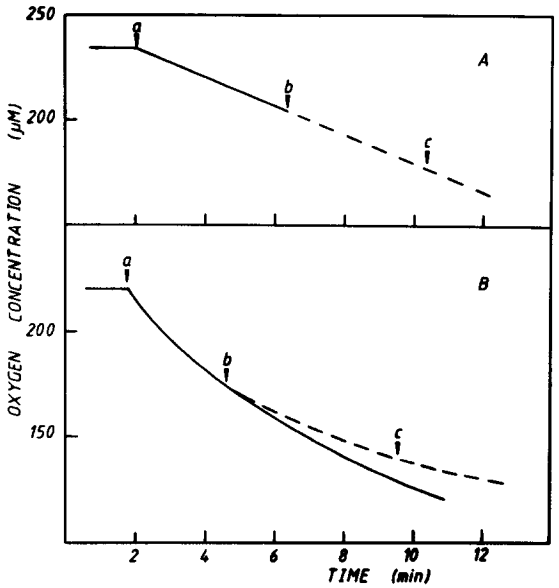

Fig. 4. The effect of aponeurocuprein on the oxidase activity of dopamine β-monooxygenase. A. The oxidation of catechol (40 mM) (a) - in the presence of 1.6 μmoles of the enzyme (——), (b) and (c) - after the additions of 48 μmoles or 96 μmoles of aponeurocuprein, respectively (- - -). B. The oxidation of dopamine (40 mM) (a) - in the presence of 0.4 μmoles of the enzyme (——), (b) and (c) - after the additions of 12 μmoles or 24 μmoles of aponeurocuprein, respectively (- - -).

DISCUSSION

The first indication of the presence of an endogenous inhibitor of dopamine β-monooxygenase in tissues was obtained as early as in 1960 (18). In subsequent years several inhibitors of dopamine β-monooxygenase were described in different tissues and plasma (19-29). It was established that the effect of some of these inhibitors was diminished in the presence of copper (20-22, 25, 29). Thus, the elimination of the inhibitory capacity of endogenous inhibitors was observed after addition of copper to homogenates or partially purified fractions of adrenal medulla, brain and heart.

Our observations that the extremely acidic protein from brain and chromaffin granules of adrenal medulla (neurocuprein) has the strong inhibiting effect on the hydroxylating activity of dopamine β-monooxygenase when the apoform of the protein was tested and the absence of the effect when it was tested in the holoform (i.e. in the copper-containing form) support above mentioned earlier findings. At the same time, as neurocuprein used was a homogeneous preparation, it gave us the possibility to observe two effects: the inhibition of dopamine β-monooxygenase after the removal of copper from neurocuprein and, vice versa, the elimination of the inhibition after the inclusion of copper in the apoprotein. Thus, the inhibiting property of neurocuprein depends on its copper content.

As it follows from the data obtained, the mechanism of the inhibition by aponeurocuprein seems to be connected with the chelation of copper from the enzyme. If the results obtained might be extrapolated to in vivo conditions the activity of dopamine β-monooxygenase should dramatically depend on the copper supply in tissues. In the case of low level of tissue copper the drop of the activity is expected as a result of the low saturation of dopamine β-monooxygenase itself by copper and, at the same time, the inc-

rease of the inhibiting capability of neurocuprein which under these conditions should be predominantly in its apoform. On the other hand, in the case of high level of tissue copper, neurocuprein is saturated with copper and therefore the inhibiting capacity of the protein is minimal. At the same time, under these conditions the saturation of dopamine β-monooxygenase with copper, and hence the activity of this copper enzyme, should be maximal. Possible physiological consequences of these extreme copper-rich and copper-poor situations have been considered in (30, 31). Another conclusion from the data obtained is the independence of the oxidase function of dopamine β-monooxygenase on the neurocuprein saturation by copper. Although both hydroxylase and oxidase functions of the enzyme depend on copper, only the hydroxylase activity depends also on the presence of reducing cosubstrates such as ascorbate, ferrocyanide and so on. Therefore it is reasonable to suggest that the inhibiting capacity of aponeurocuprein depends on the state of copper in the enzyme.

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