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NEUROCATIN-INDUCED INHIBITION OF MONOAMINE OXIDASE A IN RAT BRAIN SYNAPTOSOMES

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Abstract—Neurocatin is a small (about 2000 Da) neuroregulator isolated from mammalian brain. Earlier it was shown that addition of nanomolar concentrations of neurocatin to synaptosomes isolated from rat brain increased levels of serotonin and decreased catabolism of serotonin to 5-hydroxyindoleacetic acid (Fernandez-Novoa L and Pastuszko A, *Neurosci Lett* **122**: 83–86, 1991). In the present study, we report that neurocatin addition resulted in a striking inhibition of monoamine oxidase A activity. This inhibition became statistically significant at a neurocatin concentration of approximately 5 nM and was significant at all higher neurocatin concentrations. Neurocatin at approximately 50 nM inhibited monoamine oxidase A activity by about 90%. The inhibitory effect of neurocatin on monoamine oxidase required its incubation with intact synaptosomes since addition after breaking the synaptosomes by hypotonic buffer or lysis by Triton X-100 almost completely blocked the inhibitory effect. Measurements of the kinetic parameters of the enzyme in lysates prepared from synaptosomes incubated with neurocatin showed a decrease in V_{max} with no change in K_m for the substrate (serotonin) compared to controls. Incubation of the synaptosomes with approximately 25 nM neurocatin resulted in an 80% decrease in the V_{max} of monoamine oxidase A. Evidence that neurocatin is a powerful endogenous modulator of monoamine oxidase activity is particularly intriguing. This enzyme plays a major role in catabolism of the biogenic amines and is believed to contribute to several important neurological disorders.

In the central nervous system, as well as in peripheral tissues, oxidation of catecholamines and serotonin by monoamine oxidase (MAO†, monoamine O_2: oxidoreductase, EC 1.4.3.4) is one of the most important mechanisms for the inactivation of these biogenic amines. Monoamine oxidase exists in two forms, MAO-A and MAO-B. The A form is characterized as being more sensitive to inhibition by clorgyline and has substrate preference for norepinephrine and serotonin. The B form is characterized as being more sensitive to inhibition by deprenyl and preferentially oxidizing β-phenylethylamine and benzylamine. However, these two forms of the enzyme can also have overlapping substrate specificities [1–5].

Several studies have suggested the existence of endogenous mechanisms for the regulation of monoamine oxidase activity. Murphy and Wyatt [6] reported a reduction in monoamine oxidase activity in blood platelets from schizophrenic patients. Berrettini *et al.* [7] and Murphy *et al.* [8, 9] reported that the measured kinetic parameters of the enzyme from chronic schizophrenics show a decrease in the maximal activity of the enzyme (V_{max}), consistent with the existence of endogenous inhibitors of platelet monoamine oxidase in this disease. In 1978, Berrettini and Vogel [10] presented evidence for the endogenous inhibitor of platelet MAO in chronic schizoprenia. Similarly, Becker and Giambalvo [11] reported that inhibition of MAO from schizophrenic patients was associated with the presence in plasma of one or more proteins with low molecular weight. Endogenous MAO modulators have been also found in heart cytosol [12], urine [13, 14], cerebrospinal fluid [15, 16] and plasma [17].

In the present paper, we present evidence that an agent recently purified from mammalian brain [18] and named neurocatin inhibits the activity of rat brain monoamine oxidase A.

MATERIALS AND METHODS

Male Sprague–Dawley rats (approximately 200 g) were used throughout the study.

Preparation of synaptosomal fraction. Synaptosomal fraction was prepared from homogenates of combined cerebral hemispheres and brain stem as described by Booth and Clark [19]. The final synaptosomal pellet was suspected at approximately 10 mg protein/mL in modified Krebs–Henseleit buffer (140 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 10 mM Tris–Hepes, 1 mM Na_2HPO_4, pH 7.4) containing 10 mM glucose and 1 mM $CaCl_2$. Incubations of synaptosomes were carried out for 5–40 min at 37° under aerobic conditions.

Preparation of neurocatin. The neurocatin used during this study was obtained after elution from a semipreparative column (last step before final purification). Neurocatin in this fraction was 25–40% of the total peptide as measured by the absorption at 220 nm. The purified preparation contained approximately 57–60 μg of neurocatin/mL. The concentration of neurocatin used was estimated because the exact structure and molecular weight are not established yet. The calculated concentrations of

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† Abbreviation: MAO, monoamine oxidase.

neurocatin were based on a molecular weight of 2000 Da.

Some experiments were carried out using maximally purified neurocatin (after elution from the final analytical HPLC column) in order to verify that the inhibition of MAO is associated with this compound.

Measurement of MAO activity in intact and broken synaptosomes. Monoamine oxidase A activity was determined by the method of Wurtman and Axelrod [20]. Samples containing approximately 2 mg of synaptosomal protein in a final volume of 200 μL were incubated with 100 μM [^{3}H]serotonin (New England Nuclear, Boston, MA; 12.5 Ci/mmol) and 1 μM deprenyl (inhibitor of MAO-B). The effect of neurocatin was tested by adding to the incubation medium approximately 0 (control) and 5–50 nM neurocatin and incubating for 5- to 40-min intervals at 37°. Following this incubation, the assay mixtures were acidified with citric acid and the deaminated products were extracted by shaking with 1 mL of a toluene: ethyl acetate mixture. After centrifugation to clearly separate the two phases, 400 μL of the organic layer were transferred to a scintillation vial, 4 mL of Liquiscint scintillation fluid were added, and samples were counted in a Searle Delta 300 liquid scintillation counter. The effect of addition of neurocatin on MAO-A activity was also measured in synaptosomes in which the outer (plasma) membrane was broken. In this case, the synaptosomes were either suspended in hypotonic medium (30 mM phosphate buffer, pH 7.4) and homogenized with a Polytron or suspended in Krebs buffer with 0.2% Triton X-100. Neurocatin was added to the sample in the concentrations given above, and the activity of the enzyme was measured by incubation for 30 min with substrate as described above.

Measurement of the kinetic parameters of MAO-A. Intact synaptosomes were incubated for 5 min at 37° with 1 μM deprenyl and with 0, 12.5 or 25 nM neurocatin. After this treatment, Triton X-100 was added in a final concentration of 0.2%. The K_m and V_{max} of the enzyme were determined by incubating the lysed synaptosomes with different concentrations of [^{3}H]serotonin (40–300 μM) and incubating for 3 min at 37°. The reactions were terminated as described above, and the amount of deaminated product was measured.

Measurements of protein. Protein concentration was determined by the method of Lowry *et al.* [21] with bovine serum albumin as the standard.

Statistical analysis. All data are presented as means ± SEM. The results are compared using the paired *t*-test. When the data are presented as percent of control, the P values were calculated for the raw data.

RESULTS

Effect of neurocatin on the activity of monoamine oxidase A in intact and broken synaptosomes. Activity of MAO-A in intact synaptosomes was measured during different times of incubation (from 5 to 40 min). Twenty nanomolar neurocatin decreased the activity of the enzyme by about 50% in all measured intervals. The inhibition became statistically significant ($P < 0.05$) by 5 min (first measured time point) and it was significant at all longer times of incubation.

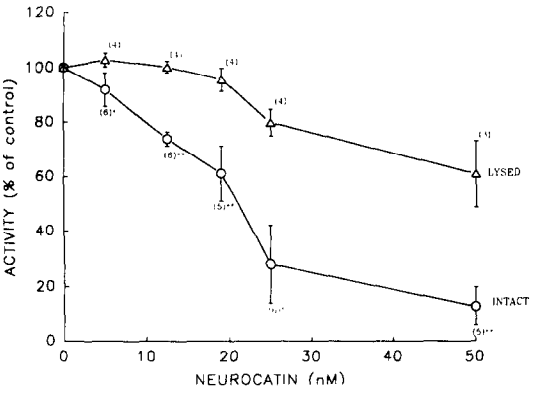

Fig. 1. Monoamine oxidase A activity in intact and broken synaptosomes as a function of neurocatin concentration. Synaptosomes (intact or broken by hypotonic buffer) were incubated with 0 (control) or from 5 to 50 nM neurocatin for 30 min at 37°. Following the incubation, aliquots were acidified, the deaminated products were extracted with toluene:ethyl acetate, and radioactivity was measured. Data are the means ± SEM for the number of experiments shown in parentheses. Each measurement was compared to its control and a paired *t*-test was used to test for statistically significant differences. Control activity of MAO-A in intact synaptosomes was 573 ± 73 pmol/mg protein/30 min, whereas in broken synaptosomes it was 1390 ± 192 pmol/mg protein/30 min. Key: $^*P < 0.05$ and $^{**}P < 0.001$ vs control.

The dependence of monoamine oxidase A activity on the concentration of neurocatin in intact and broken synaptosomes is shown in Fig. 1. The MAO-A activity in intact synaptosomes decreased with increased concentration of neurocatin, the decrease becoming statistically significant at approximately 5 nM ($P < 0.05$). Half-maximal effect was observed at approximately 20 nM neurocatin. Incubation of synaptosomes with 40–50 nM neurocatin inhibited MAO-A activity by about 90%. The inhibitory effect of neurocatin on monoamine oxidase required the neurocatin to be incubated with intact synaptosomes. Suspending the synaptosomes in hypotonic phosphate buffer and homogenizing with a Polytron prior to addition of neurocatin almost completely blocked the inhibitor effect (Fig. 1). Identical results were obtained when the synaptosomes were lysed by Triton X-100 in a concentration of 0.2%. Under these conditions, we did not observe any changes in the activity of MAO-A caused by the treatment with Triton X-100. In broken synaptosomes, addition of 50 nM neurocatin caused decreased activity of the enzyme by only approximately 25% (not statistically significant). It cannot be excluded that the small residual inhibition was due to the presence of a small fraction of intact synaptosomes or "right side out" vesicles formed from synaptosomes.

To determine if there is any time dependence of the inhibition, assessed by preincubating the synaptosomes with neurocatin for different times prior to addition of substrate, synaptosomes were preincubated with 20 nM neurocatin for 1, 2.5, 5, 10

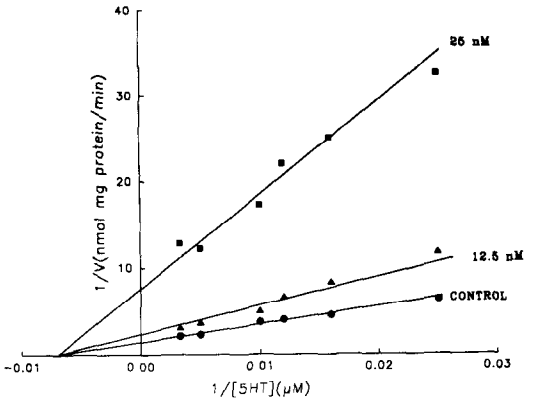

Fig. 2. Inhibition of monoamine oxidase A activity from rat synaptosomes by neurocatin. The synaptosomes were incubated for 5 min without (control) and with 12.5 or 25 nM neurocatin. After this treatment, Triton X-100 was added and the activity of MAO-A was measured as described in Materials and Methods. The concentrations of serotonin (5-HT) used throughout the study were from 40 to 300 μM. Data presented are for a representative experiment.

and 20 min, and then the fraction was broken, and the activity of MAO-A measured as described in Materials and Methods. It was observed that the inhibitory effect was complete and already maximal (53%) with a 1-min preincubation of the synaptosomes with neurocatin, and it was constant at all longer times of incubation.

Effect of neurocatin on kinetic parameters of synaptosomal MAO-A. The dependence of the rate of the monoamine oxidase reaction on the concentration of substrate (serotonin) was measured in lysates of suspensions of intact synaptosomes incubated for 5 min with 0 (control), 12.5 and 25 nM neurocatin. The results are shown in Fig. 2. Neurocatin decreased the maximal rate (V_{max}) of monoamine oxidase A with no significant changes in the affinity (K_M) for the substrate (serotonin) compared to control. The extent of the change in V_{max} was dependent on the concentration of neurocatin used; 12.5 nM neurocatin decreased V_{max} by about 30%, while 25 nM neurocatin caused a decrease of about 80% (Table 1).

Table 1. Kinetic parameters of MAO-A in rat brain synaptosomes in the presence of neurocatin

Conditions	K_m (μM)	V_{max} (nmol/mg protein/min)
Control	117.8 ± 2.8	0.57 ± 0.04
Neurocatin		
12.5 nM	141.3 ± 7.4	0.42 ± 0.023*
25 nM	136.4 ± 12.4	0.13 ± 0.011†

Synaptosomes were preincubated for 5 min without (control) and with 12.5 and 25 nM neurocatin. Aliquots were then treated with Triton X-100 and activity of MAO-A was measured as described in Materials and Methods. Values are means ± SEM for three experiments.

* $P < 0.05$ vs control.

† $P < 0.02$ vs control.

DISCUSSION

The importance of catecholamines and their catabolism in normal function of the brain has generated an intense interest in the regulation of monoamine oxidase activity. Endogenous monoamine oxidase modulators have been reported as being found in the cytosol of various tissues, in blood plasma, in urine, and in the cerebrospinal fluid (see beginning of paper). Recently, Egashira *et al.* [22] reported also the existence of an endogenous factor in a monkey brain extract which is a potent inhibitor of monoamine oxidase activity of monkey brain *in vitro*. These endogenous monoamine oxidase inhibitors have been reported to have many different molecular weights. Glover *et al.* [13], for example, demonstrated the existence, in human urine, of small compounds with a molecular weight of approximately 180 which were monoamine oxidase inhibitors. Normal human cerebrospinal fluid has been reported to contain compounds of molecular weights in the range of 3,000 to 35,000, which act as MAO inhibitors [16]. Egashira *et al.* [15] reported that dog cerebrospinal fluid contains peptides of less than 5000 molecular weight which are inhibitors of both MAO-A and -B. Monkey brain contains MAO-A and -B inhibitors, one with a molecular weight <1350; the other, with peptide properties, with a molecular weight of about 2500 [22]. Some of the endogenous compounds can act as activators or inhibitors of monoamine oxidase, depending on the substrate used and the animal species [15, 23]. In all cases these compounds acted directly on the monoamine oxidase enzyme *in vitro*.

In the present paper we have shown that neurocatin, a neuroregulatory factor isolated from bovine brain, inhibits monoamine oxidase A activity in isolated presynaptic nerve terminals (synaptosomes). Neurocatin has been purified to a single chromatographic peak by high resolution HPLC [18]. It appears to be a small peptide with a molecular weight in the range of 2000–2500 Da. This compound has been shown to increase the level of serotonin in, and its release from, synaptosomes. In addition, it decreases the level of 5-hydroxyindoleacetic acid, the major product of serotonin catabolism [24]. These observations indicated that neurocatin could be an inhibitor of monoamine oxidase activity. The results in the present work are consistent with this suggestion. In synaptosomes from rat brain, neurocatin decreased the activity of MAO-A in a concentration- and time-dependent manner. A very interesting aspect of the inhibitory effect on MAO-A was that it occurred only when neurocatin was incubated with intact synaptosomes. The effect was abolished almost completely when the synaptosomes were broken by Triton X-100 or by homogenization in hypotonic medium prior to neurocatin addition. Measurements of the kinetics of the monoamine oxidase activity indicated that the inhibition is associated with a decrease in the V_{max}, with no significant change in the apparent affinity (K_m) of the enzyme for the substrate (serotonin).

Numerous investigators have described the effects of endogenous MAO inhibitors on the kinetic parameters of the enzyme. Some of these compounds caused noncompetitive inhibition with a decrease

in the V_{max} of the reaction [10, 11, 13, 15–17, 22]. However, in contrast to the other reported affectors of MAO activity, neurocatin did not have a direct effect on the enzyme. The inhibitory effect of neurocatin on MAO-A was largely abolished by agents which ruptured the synaptosomal membrane, whereas the other agents were fully active in the lysed preparations.

Neurocatin thus appears to be a new endogenous modulator of monoamine oxidase activity. The modulation is time dependent and is retained when the synaptosomes are lysed. Such a modulation is consistent with covalent modifications, such as phosphorylation, of the enzyme. This modification could be produced, for example, by protein kinases acting in response to activation of neurocatin receptors in the synaptosomal membrane. The ability of neurocatin to induce rapid and large changes in the activity of MAO is consistent with its playing an important role in the normal function of the brain. It also raises the possibility that neurocatin is a factor in some types of pathology of the brain.

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REFERENCES

1. Johnston JP, Some observations upon a new inhibitor of monoamine oxidase in brain tissue. *Biochem Pharmacol* **17**: 1285–1297, 1968.
2. Fowler CJ and Tipton KF, Deamination of 5-hydroxytryptamine by both forms of monoamine oxidase in the rat brain. *J Neurochem* **38**: 733–736, 1982.
3. Garrick NA and Murphy DL, Monoamine oxidase type A: Differences in selectivity towards *l*-norepinephrine compared to serotonin. *Biochem Pharmacol* **31**: 4061–4066, 1982.
4. Fowler CJ and Tipton KF, On the substrate specificities of the two forms of monoamine oxidase. *J Pharm Pharmacol* **36**: 111–115, 1984.
5. Kinemuchi H, Wakui Y and Kamijo K, Substrate selectivity of type A and type B monoamine oxidase in rat brain. *J Neurochem* **35**: 109–115, 1980.
6. Murphy DL and Wyatt RJ, Reduced monoamine oxidase activity in blood platelets from schizophrenic patients. *Nature* **238**: 225–226, 1972.
7. Berrettini WH, Vogel WH and Clouse R, Platelet monoamine oxidase in chronic schizophrenia. *Am J Psychiatry* **134**: 805–806, 1977.
8. Murphy DL, Belmaker R and Wyatt RJ, Monoamine oxidase in schizophrenia and other behavioral disorders. *J Psychiatr Res* **11**: 221–248, 1974.
9. Murphy DL, Donnelly CH, Miller L and Wyatt RJ, Platelet monoamine oxidase in chronic schizophrenia. *Arch Gen Psychiatry* **33**: 1377–1381, 1977.
10. Berrettini WH and Vogel WH, Evidence for an endogenous inhibitor of platelet MAO in chronic schizophrenia. *Am J Psychiatry* **135**: 605–607, 1978.
11. Becker RE, and Giambalvo CT, Endogenous modulation of monoamine oxidase in schizophrenia and normal humans. *Am J Psychiatry* **139**: 1567–1570, 1982.
12. Ichikawa K, Hashizume K and Yamada T, Monoamine oxidase inhibitory modulators in rat heart cytosol: Evidence for induction by thyroid hormone. *Endocrinology* **111**: 1803–1809, 1982.
13. Glover V, Reveley MA and Sandler M, A monoamine oxidase inhibitor in human urine. *Biochem Pharmacol* **29**: 467–470, 1980.
14. Glover V, Bhattacharya SK and Sandler M, Benzodiazepines reduce stress-augmented increase in rat urine monoamine oxidase inhibitor. *Nature* **292**: 347–349, 1981.
15. Egashira T, Takano R and Yamanaka Y, Demonstration of endogenous inhibitors of monoamine oxidase in dog cerebrospinal fluid. *Jpn J Pharmacol* **42**: 583–586, 1986.
16. Becker RE, Giamvalvo CT, Fox RA and Macho M, Endogenous inhibitors of monoamine oxidase present in human cerebrospinal fluid. *Science* **221**: 476–478, 1983.
17. Giambalvo CT and Becker RE, Modulators of monoamine oxidase in plasma *Life Sci* **29**: 2017–2024, 1981.
18. Yee DK, Pastuszko P, Pastuszko A and Wilson DF, Purification of neurocatin, a neuroregulatory factor from brain. *Neurosci Lett* **114**: 213–219, 1990.
19. Booth RFG and Clark JB, A rapid method for the preparation of relatively pure metabolically competent synaptosomes from rat brain. *Biochem J* **176**: 365–370, 1978.
20. Wurthman RJ and Axelrod J, A sensitive and specific assay for the estimation of monoamine oxidase. *Biochem Pharmacol* **12**: 1439–1441, 1963.
21. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
22. Egashira T, Obata T, Nagai T, Kimba Y, Takano R and Yamanaka Y, Endogenous monoamine oxidase inhibitor-like substances in monkey brain. *Biochem Pharmacol* **38**: 597–602, 1989.
23. Yu PH and Boulton AA, Activation of platelet monoamine oxidase by plasma in the human *Life Sci* **25**: 31–36, 1979.
24. Fernandez-Novoa L and Pastuszko A, Neurocatin induces changes in release and level of serotonin in synaptosomal fraction from rat brain. *Neurosci Lett* **122**: 83–86, 1991.