Vol. 116, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 31, 1983 Pages 735-742

A NEW FEATURE OF ANGIOTENSIN-CONVERTING ENZYME IN THE BRAIN: HYDROLYSIS OF SUBSTANCE P

Hideyoshi Yokosawa, Shogo Endo, Yasuhiko Ogura and Shin-ichi Ishii

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

Received September 20, 1983

SUMMARY: Highly purified rat brain angiotensin-converting enzyme hydrolyzes substance P which contains a C-terminal amino acid with an amidated carboxyl group. The hydrolysis of substance P verified by amino-group fluorometry and by high-performance liquid chromatography is inhibited by captopril, but not by phosphoramidon. The presence of sodium chloride is essential for the hydrolysis. The analyses of cleavage products indicate that the enzyme hydrolyzes substance P between Phe7-Phe8 and Phe8-Gly9 by an endopeptidase action, followed by successive release of dipeptides by a dipeptidyl carboxypeptidase action.

Angiotensin-converting enzyme (ACE, EC 3.4.15.1) is a dipeptidyl carboxypeptidase which catalyzes the generation of angiotensin II from angiotensin I, releasing the C-terminal dipetide, His-Leu (1,2). The ACE activity has first been reported to be present in the brain by Yang and Neff (3). Subsequent studies on the regional distribution of ACE activity or immunoreactivity in the brain (3-14) indicated that a positive correlation exist between the contents of ACE and those of other components of renin-angiotensin system (15) in some areas of the brain, whereas such a correlation cannot be found in other areas of the brain, e.g, the substantia nigra.

In a previous communication (16), we have reported the purification of ACE from rat brain and its inhibition by various neuropeptides. The purified enzyme was strongly inhibited by

Abbreviations: ACE, angiotensin-converting enzyme; HPLC, high-performance liquid chromatography.

0006-291X/83 $1.50

bradykinin, enkephalin analogue and substance P. The susceptibility of the ACE toward various neuropeptides and the correlation between distribution of ACE and of cognate neuropeptides prompted us to propose that ACE not only plays a role in the brain renin-angiotensin system through endogenous formation of angiotensin II but also participates in the metabolism of neuropeptides other than angiotensin.

In the course of further studies on the role of ACE in the brain, we found a new feature of ACE in the brain; it effectively hydrolyzed substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), although this peptide lacked a free terminal carboxyl group. Our finding fits well with the above proposition for the role of ACE in the brain.

MATERIALS AND METHODS

Rat brain ACE was purified as previously described (16). ACE-I preparation was mainly used in this communication, because almost the same results were obtained with ACE-II preparation.

Substance P, (Tyr^8)-substance P, and phosphoramidon were purchased from the Peptide Institute, Osaka. Substance P octapeptide, fragment (4-11), and Phe-Phe were obtained from Sigma Chemical Co., St. Louis, Mo. Fluorescamine was a product of F. Hoffmann-La Roche Inc., Diagnostica. Captopril was kindly provided by Dr. A. Awaya of Mitui Pharmaceuticals, Inc., Tokyo.

The reaction mixture (100 µl) contained 0.1 M borate buffer (pH 8.2), 0.3 M NaCl, 50 µM substance P, and 33 ng ACE and was incubated at 37°C. The extent of hydrolysis was monitored by measuring fluorescence generated by the reaction of amino groups with fluorescamine (17) and by HPLC as previously described (18). The chromatographic equipment consisted of a TRI ROTAR pump, a VL-611 sample injector, a GP-A30 solvent programer, and a UVI-DEC-100 spectrophotometer (JAPAN Spectroscopic Co. Ltd., Tokyo). The column used was TSK-LS-410-ODS-SIL (4 x 300 mm)(Toyo Soda Kogyo, Ltd., Tokyo).

Cleavage sites of substance P were determined by the following two procedures: (a) amino acid analyses on a JEOL JLC-6AH amino acid analyzer of the cleavage products separated by HPLC, and (b) identification of dansylated derivatives of N-terminal amino acid residues newly formed as a result of hydrolysis, as previously described (16).

RESULTS

Hydrolysis of substance P with the ACE was measured fluorometrically. As shown in Fig. 1, the generation of fluorescence (amino groups) was proportional to time and was

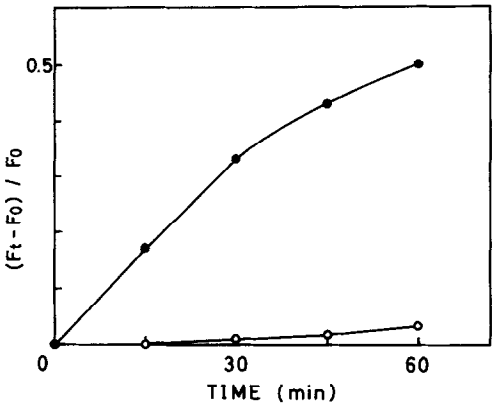

Fig. 1. Hydrolysis of substance P with ACE in the presence (o) and absence (•) of 1 μM captopril, as monitored by measuring fluorescence generated by the reaction of amino groups with fluorescamine. The vertical coordinate indicates (Ft-Fo)/Fo, where Ft represents fluorescence measured at a given reaction time and Fo indicates the initial fluorescence.

completely inhibited by 1 μM captopril. Hydrolysis of substance P analogues, (Tyr^8)-substance P and substance P octapeptide, gave almost the same results. A concentration of approximately 10 nM of captopril was required for 50% inhibition. Phosphoramidon at a concentration of 10 μM exhibited negligible inhibitory activity. The presence of sodium chloride was essential for the hydrolysis of substance P, in a manner similar to that for the hydrolysis of a typical synthetic substrate, hippuryl-His-Leu (16). The rate of amino group generation from substance P was comparable to those from bradykinin and Leu-enkephalin-(Arg^6) treated with the same enzyme preparation (16).

Five major peptide-containing peaks were detectable by HPLC in the reaction mixture of substance P and ACE (Fig. 2). The peptide materials in peaks 4 and 5 coeluted with standards Phe-Phe and substance P, respectively. The area of peak 5, substance P, decreased as a function of time in an inverse manner to the increase of fluorescence described above (Fig. 1), while those of peaks 1, 2 and 4 increased as a function of time (Fig. 2(a)-(c)). As shown in Fig. 2(d), captopril (1 μM) completely

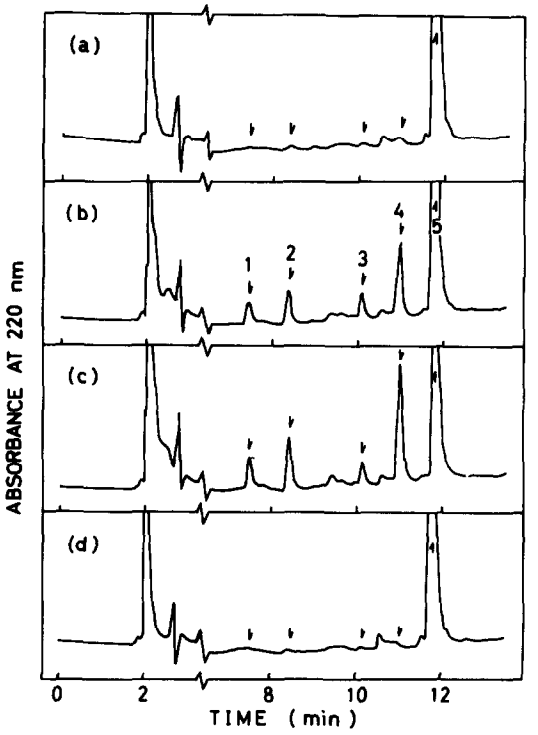

Fig. 2. High-performance liquid chromatography analysis of products of ACE digestion of substance P. The reaction mixture was incubated at 37°C for 0 (a), 15 (b), and 30 min (c) in the absence of captopril and for 30 min (d) in the presence of 1 μM captopril, and 40 μl-aliquots were analyzed by HPLC using a TSK-LS-140-ODS-SIL column (4 x 300 mm). The column was equilibrated with acetonitrile/50 mM H_3PO_4, pH 1.9, (5 : 95, V/V). Elution was carried out at room temperature with a 16 min linear gradient of 5-65% acetonitrile in 50 mM H_3PO_4, pH 1.9, at a flow rate of 1 ml/min. The absorbance at 220 nm was monitored.

inhibited the hydrolysis of substance P with ACE. Amino acid compositions of cleavage products separated by HPLC are shown in Table I. The results allowed the assignment of these isolated products. The detection of peptide fragments (1-7), (1-8) and (9-11) indicates that ACE can cleave substance P at Phe^7-Phe^8 and Phe^8-Gly^9 bonds by its endopeptidase-like action. The latter bond seems to be more susceptible than the former one, because the amount of peptide fragment (9-11) was almost comparable to that of substance P hydrolyzed. Fragment (7-8), Phe-Phe, appears to be generated from fragment (1-8) by the dipeptidyl carboxypeptidase activity of ACE. Although fragment (8-11) has

TABLE I
Amino Acid Compositions of Cleavage Products of Substance P[a]

Peak	Amino Acid (Mol %)								Fragment Identified	Yield (%)[b]
	Arg	Pro	Lys	Glu	Phe	Gly	Leu	Met		
1	14	27	15	29	13	2	0	0	(1-7)	8
2	0	0	0	1	0	35	36	28	(9-11)	89
3	12	30	13	21	23	0	0	0	(1-8)	2
4	0	0	0	0	100	0	0	0	(7-8)	86
5									Complete	

[a]The extent of hydrolysis of substance P was 66%.
[b]Determined on the basis of the amount of substance P hydrolyzed.

not been detected, the fact that the generation of fragment (9-11) is completely inhibited by 1 μM captopril supports the endopeptidase activity of ACE toward substance P.

N-termini newly formed as the result of hydrolysis of substance P with ACE were determined to be in the following order; Phe≥Gly>Gln>>Lys, indicating the successive cleavages of fragment (1-8) and, possibly, of fragment (1-7) by the dipeptidyl carboxypeptidase action, as well as the endopeptidase action toward substance P as described above.

DISCUSSION

We described a new feature of ACE in the brain. Substance P was hydrolyzed with the purified rat brain ACE, although it contained a C-terminal amino acid with an amidated carboxyl group. ACE is characterized as dipeptidyl carboxypeptidase (1, 2) and the endopeptidase activity of ACE has not been reported except for a recent report on the hydrolysis with human kidney ACE of fluorogenic substrate containing C-terminal nitrobenzylamine (19). The possibility that the hydrolysis of substance P is due to a contaminant in our ACE preparation is ruled out by the fact that the hydrolysis of substance P and, especially, the generation of a fragment, Gly-Leu-Met-NH_2, from substance P are

completely inhibited by captopril, a specific inhibitor of ACE. Furthermore, the presence of sodium chloride is essential for the activity of ACE toward substance P, in a manner simialr to that toward most typical substrate (1,2). Phosphoramidon, a specific inhibitor for neutral metalloendopeptidase having dipeptidyl carboxypeptidase activity (20), does not inhibit the activity of ACE either toward substance P or toward hippuryl-His-Leu (16).

By the action of ACE, substance P was initially hydrolyzed at the peptide bond between Phe^{8}-Gly^{9} or Phe^{7}-Phe^{8} and the liberated fragments (1-8) and (1-7) were further cleaved sequentially, releasing dipeptides. The demonstration of good susceptibility of substance P toward ACE, whose substrate has been thought to require a free C-terminal carboxyl group, leads us to suppose that ACE has an intrinsic endopeptidase activity and exerts this activity enhanced under some circumstances even if the substrates have no terminal free carboxyl groups. The conformation of substance P around scissile bonds may play an important role in enhancing the endopeptidase activity of ACE. Since various neuropeptides with amidated carboxyl termini are present in the brain (21), it can be thought that ACE may involve also in the metabolism of neuropeptides lacking free carboxyl termini, depending on their accessibility to the active site of the enzyme.

Substance P-degrading enzyme has been reported to be purified from human brain (18). The enzyme was inhibited by metal-chelating agents but not either by captopril or phosphoramidon. The cleavage sites were between Gln^{6}-Phe^{7}, Phe^{7}-Phe^{8} and Phe^{8}-Gly^{9}. Another enzyme that hydrolyzes substance P in pig caudate has been recently reported to be identical with a membrane-bound neutral metalloendopeptidase susceptible to phosphoramidon

(22). The cleavage sites were between Gln^6-Phe^7, Phe^7-Phe^8 and Gly^9-Leu^{10}. The molecular weights of the two enzymes were smaller than that of ACE. Differences in cleavage site, inhibitor susceptibility, and molecular weight exist between ACE and the enzymes which have been reported to hydrolyze substance P in the brain (18,22). To elucidate whether ACE involves in the metabolism of substance P in a specific area of brain, where substance P is distributed at the high concentration, further studies are now in progress.

ACKNOWLEDGMENT

This study was supported in part by a research grant from the Ministry of Education of Japan.

REFERENCES

1. Soffer, R.L. (1976) Annu. Rev. Biochem. 45, 73-94.
2. Erdös, E.G. (1979) Handbook of Experimental Pharmacology, 25, Suppl., pp 427-487, Springer, Heidelberg.
3. Yang, H.-Y.T., and Neff, N.H. (1972) J.Neurochem. 19, 2443-2450.
4. Yang, H.-Y.T., and Neff, N.H. (1973) ibid. 21, 1035-1036.
5. Poth, M.M., Heath, R.G., and Ward, M. (1975) ibid. 25, 83-85.
6. Ijic, R.P., Robinson, C.J.G., and Erdös, E.G. (1977) Central Action of Angiotensin and Related Hormones, pp 23-27, Pergamon Press, New York.
7. Arregui, A., Emson, P.C., and Spokes, E.G. (1978) Eur. J. Pharmacol. 52, 121-124.
8. Arregui, A., and Iversen, L.L. (1978) ibid. 52, 147-150.
9. Rix, E., Ganten, D., Schüll, B., Unger, Th., and Taugner, R. Neurosci. Lett. 22, 125-130.
10. Chevillard, C., and Saavedra, J.M. (1982) J. Neurochem. 38, 281-284.
11. Saavedra, J.M., and Chevillard, C. (1982) Neurosci. Lett. 29, 123-127.
12. Saavedra, J.M., Fernandez-Pardal, J., and Chevillard, C. (1982) Brain Res. 245, 317-325.
13. Brownfield, M.S., Reid, I.A., Ganten, D., and Ganong, W.F. (1982) Neuroscience 7, 1759-1769.
14. Defendini, R., Zimmerman, E.A., Weare, J.A., Alhenc-Gelas, F., and Erdös, E.G. (1983) Neuroendocrinology 37, 32-40.
15. Printz, M.P., Ganten, D., Unger, T., and Phillips, M.I. (1982) The Renin-Angiotensin System in the Brain, pp 3-52, Springer, Berlin.
16. Yokosawa, H., Ogura, Y., and Ishii, S. (1983) J. Neurochem. 41, 403-410.
17. Udenfriend, S., Stein, S., Böhlen, P., Dairman, W., Leimgruber, W., and Weigele, M. (1972) Science 178, 871-872.
18. Lee, C.-M., Sandberg, B.E.B, Hanley, M.R., and Iversen, L.L. (1981) Eur. J. Biochem. 114, 315-327.

19. Hersh, L.B., Gafford, J.T., Powers, J.C., Tanaka, T., and Erdös, E.G. (1983) Biochem. Biophys. Res. Commun. 110, 654-659.
20. Fulcher, I.S., Matsas, R., Turner, A.J., and Kenny, A.J. (1982) Biochem. J. 203, 519-522.
21. Klee, W.A. (1979) Adv. Protein Chem. 33, 243-286.
22. Matsas, R., Fulcher, I.S., Kenny, A.J., and Turner, A.J. (1983) Proc. Natl. Acad. Sci. USA 80, 3111-3115.