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SUBSTANCE K AND SUBSTANCE P AS POSSIBLE ENDOGENOUS SUBSTRATES OF ANGIOTENSIN CONVERTING ENZYME IN THE BRAIN

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In the brain angiotensin converting enzyme is highly localized to a striatonigral pathway, which contains no endogenous angiotensin. Substance P, also localized to a striatonigral pathway, is degraded by ACE via two different pathways. The lung and striatal isozymes of angiotensin converting enzyme exhibit differential cleavage of substance P, with lung preferring an initial tripeptide cleavage, and striatum an initial dipeptide cleavage. Substance K is degraded by the striatal isozyme but is not cleaved by the lung isozyme. Substance P 5-11 is not cleaved by either form of angiotensin converting enzyme. © 1985 Academic Press, Inc.

Angiotensin converting enzyme (ACE, EC 3.14.5.1) functions outside the brain as a dipeptidyl carboxypeptidase, removing the carboxyl two amino acids from the decapeptide angiotensin I, transforming it to the octapeptide angiotensin II. Angiotensin II, a physiological regulator of the cardiovascular system, is formed largely by ACE in the lung (1,2). The testes (1,3) and the brain (4,5) are two other major sources of ACE in which it is not clear whether angiotensin I is the endogenous substrate. In the brain ACE is highly localized to a prominent neuronal pathway with cell bodies in the corpus striatum and axons which descend to terminate in the substantia nigra (4, 6-8). This striatonigral pathway lacks endogenous angiotensin, raising the possibility that another endogenous peptide, possibly a neurotransmitter, is synthesized or degraded by ACE in the brain.

Abbreviations: ACE, angiotensin converting enzyme; SP, substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2); SK, substance K (His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2); TFA, trifluoroacetic acid; OD, optical density

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Substance P (SP) is a major neurotransmitter peptide which is also localized to the striatonigral pathway (9,10). Recently a closely related peptide substance K (SK) has been identified in the brain (11-13). A single messenger RNA encodes for a protein containing the sequence of both SP and SK (11). Since the highest concentrations of SK are also in the substantia nigra and the corpus striatum (13), it is possible that some central neuronal pathways previously thought to utilize SP as their neurotransmitter may actually contain SK as their predominant neuropeptide.

One difficulty in suggesting that SK or SP are endogenous substrates for ACE is that both of these peptides have carboxyl terminal amide groups, and ACE had been thought incapable of cleaving such amidated peptides (2). Recently several groups have shown that ACE from rabbit lung, or rat brain or human preparations can degrade SP (14-16). In the present study we show that SP is degraded by ACE with distinct cleavage patterns in the corpus striatum of the brain and in the lung. Moreover, we show that both SK and SP are active substrates for brain ACE, while SP but not SK is degraded by lung ACE.

Materials and Methods

ACE from lung and corpus striatum was purified as described (Strittmatter, S.M., Thiele E.A., Kapiloff, M.K. and Snyder, S.H., in preparation). SP and SK were obtained from Pharmacia Fine Chemicals (Piscataway, NJ), and SP 5-11 from Beckman (Silver Spring, MD). [2-Propyl-3,4-[^{3}H](N)SP and Formula 963 scintillation fluid were obtained from New England Nuclear (Boston, MA). Captopril was obtained from Squibb and Sons, Incorporated (New York, NY). Hip-His-Leu was purchased from Sigma (St. Louis, MO), Dansyl chloride from Aldrich (Milwaukee, WI). Six N HCl for hydrolysis was obtained from Pierce (Rockford, IL).

SP, SP 5-11, and SK were incubated with lung or striatal ACE in a total volume of 0.2 ml of 100 mM Tris HCl, pH 7.7, 300 mM NaCl at 37°C. After a variable period of incubation, trifluoroacetic acid (TFA) was added to 0.5°/°, inhibiting further enzymatic reaction. Controls contained captopril in the assay mixture. Samples were then applied to an Alltech/ Applied Science C-18 Reverse Phase Column and eluted with a linear, 1 hr, 1 ml/min gradient of 0-80°/° acetonitrile in 0.1°/° TFA, while monitoring column outflow for optical density (OD), at 210 nm wavelength. HPLC fractions containing absorbant material were lyophilized for amino acid analysis. Purity of collected fractions was confirmed by chromatography on both an HPLC C-18 reverse phase column and a Waters HPLC TSK-2000 sizing column.

N-terminus analysis and amino acid composition of the degradation products for lung and striatal ACE were performed by separating dansyl-amino acids on reverse phase HPLC. For N-terminus analysis, samples were

dansylated by incubating the lyophilized peptides with 50 µl of 2 mg/ml dansyl chloride and 100 µl 0.15 M $NaHCO_3$ for 1 hr at 37°C, the mixture was lyophilized, and the dansylated peptides hydrolyzed under vacuum for 18 hr at 115°C in 6 N HCl. After a second lyophilization, the sample was resuspended in 0.1°/° TFA, and applied to an HPLC Ranin microsorb 5 cm reverse phase column at 55°C. The sample was eluted at 1 ml/min for 7 min with 8°/° acetonitrile in 0.1°/° TFA, and then a 2 hr linear gradient from 8-68°/° of acetonitrile in 0.1°/° TFA. The column eluate was mixed with isopropranol (1:2) and fluorescence of the mixture recorded continuously. The isopropanol increased the sensitivity of the system 10-fold because dansyl groups are more fluorescent in organic solvents.

To determine the amino acid composition of degradation products, fragments were hydrolyzed prior to dansylation, following procedures described above. Dansyl-amino acids in samples were identified by coincident elution with dansyl amino acid standards.

Quantitative amino acid analysis was conducted on several degradation products of SP degradation by lung ACE by Dr. Juris Ozols (Dept. Biochemistry, University of Connecticut School of Medicine).

To analyze [^{3}H]SP degradation, [^{3}H]SP (100,000 cpm, 5.6 pmoles), 10 µM SP, and lung or striatal ACE (60 ng) were incubated in 100 mM Tris HCl, pH 7.7, 300 mM NaCl for 20 min at 37°C. TFA was added to 0.5°/° to inhibit further reaction. Samples were chromatographed as described above. Fractions of the column eluate were collected at 1 min intervals, added to 10 ml Formula 963 scintillation fluid and radioactivity measured by liquid scintillation spectrometry. The radioactivity for fractions 22-24 and 29-31 was added together to obtain values for Arg-Pro and SP 1-5 respectively, and the ratio of the values is reported in the text. The results are from one of four experiments with identical results.

Lung and striatal enzyme activities were monitored using the Hip-His-Leu fluorometric assay previously described (5).

Results

To examine the pattern of SP degradation by lung ACE, we incubated SP with purified lung ACE for 4 hr and analyzed the products formed by HPLC C-18 reverse phase chromatography (Table 1, Figure 1). Eleven distinct peptide peaks occur reflecting two distinct patterns of SP degradation (Figure 2). One pattern involves an initial removal of the carboxyl terminal tripeptide followed by sequential removal of dipeptides as is evidenced by the presence of Gly-Leu-Met-NH_2, Phe-Phe, Gln-Gln, Lys-Pro, and Arg-Pro. The other pattern reflects an initial cleavage of the carboxyl terminal dipeptide followed by sequential cleavages of dipeptides in SP until Pro is in the penultimate position, rendering the peptide resistent to further ACE cleavage (17). Fragments indicating this cleavage pattern are Leu-Met-NH_2, Phe-Gly, Gln-Phe, Arg-Pro-Lys-Pro-Gln-Gln-Phe (SP 1-7), and Arg-Pro-Lys-Pro-Gln (SP 1-5).

Table 1

Composition of SP Degradation Products Formed by Lung ACE

Peak	nmoles per sample								Structure
Initial tripeptide cleavage									
	Arg	Pro	Lys	Gln	Phe	Gly	Leu	Met	
A									Gln-Gln
B	-	56	50	5	-	5	-	-	Lys-Pro
C	58	58	-	-	-	-	-	-	Arg-Pro
H	-	-	-	-	-	67	66	65	Gly-Leu-Met-NH_2
J	-	-	-	-	96	-	-	-	Phe-Phe
Initial dipeptide cleavage									
	Arg	Pro	Lys	Gln	Phe	Gly	Leu	Met	
D	9	16	7	10	-	4	-	-	Arg-Pro-Lys-Pro-Gln
E	-	-	-	-	-	-	10	10	Leu-Met-NH_2
F									Phe-Gly
G	-	-	-	8	8	1	-	-	Gln-Phe
I	4	8	4	9	4	1	-	-	Arg-Pro-Lys-Pro-Gln-Gln-Phe
K	14	28	12	29	26	14	15	14	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

Amino acid composition of products was determined as described in Materials and Methods. Quantitative analysis was performed on all peaks except A and F. Dashes indicate that insufficient quantities precluded detection. Values are the means of three determinations which varied less than 20°/∘.

Quantitative amino acid analysis shows that initial removal of the carboxyl terminal tripeptide by the lung enzyme is about five times more prevalent than cleavage of the carboxyl terminal dipeptide. In HPLC analyses at timed intervals associated with 90°/∘, 50°/∘, or 10°/∘ degradation of SP, the ratio of these two pathways is essentially the same. HPLC analysis indicates that both pathways of degradation are completely

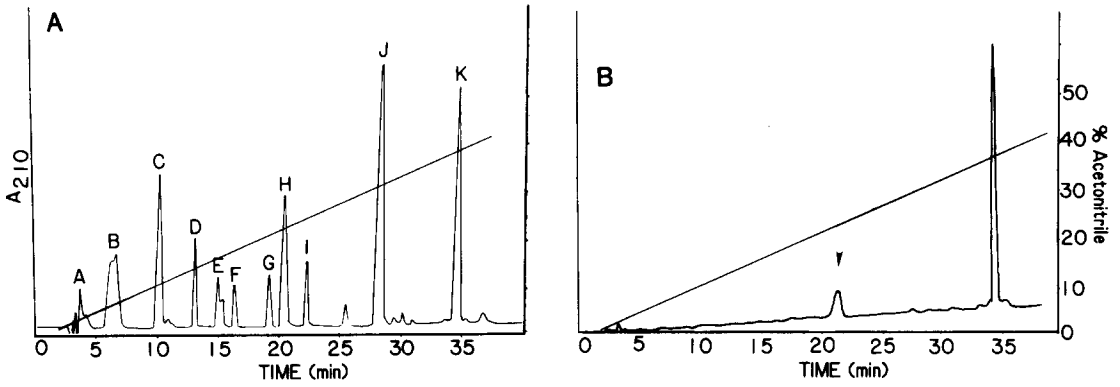

Figure 1 SP Degradation by Lung ACE

(a) SP (100 nmoles, 400 μM) was incubated with 600 ng lung ACE in 100 mM Tris HCl, pH 7.7, 300 mM NaCl for 4 hr at 37°C. Sample was applied to the column, eluted with a linear, 1 hr, 1 ml/min gradient of 0-100°/° of 80°/° acetonitrile in 0.1°/° TFA. Absorbance of the eluate was monitored with OD 210 nm and absorption peaks collected. Amino acid composition of peaks was determined as described in Materials and Methods. Peaks were identified as (A) Gln-Gln, (B) Lys-Pro, (C) Arg-Pro, (D) SP 1-5, (E) Leu-Met-NH_2, (F) Phe-Gly, (G) Gln-Phe, (H) Gly-Leu-Met-NH_2, (I) SP 1-7, (J) Phe-Phe, and (K) SP 1-11. (b) A second incubation with the addition of 10 nM captopril demonstrates complete inhibition of SP degradation (elution of captopril indicated by arrow). The data presented are representative of six experiments.

inhibited by 10 nM captopril (Figure 1) or by 0.5 mM EDTA. Once initiated, the processing of SP occurs rapidly. In analyses after only about 10°/° degradation of SP, we fail to detect the fragments SP 1-9, SP 1-8, or SP 1-6 presumably because of their rapid degradation.

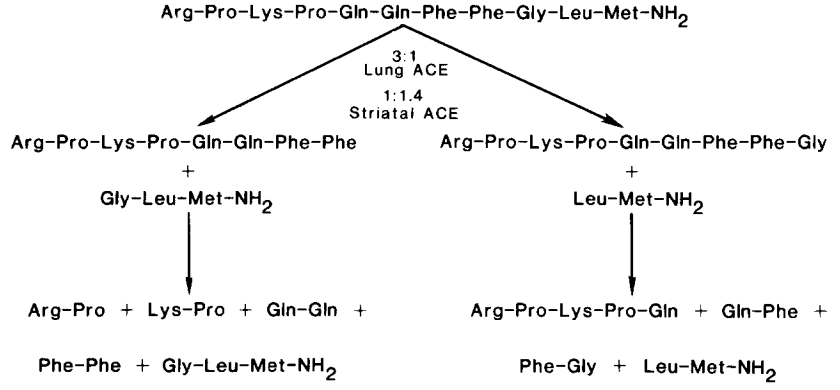

Figure 2 Two Cleavage Pathways of SP Degradation by ACE

Both lung and striatal ACE cleave SP via two different pathways, involving either initial C-terminal tripeptide cleavage or initial C-terminal dipeptide cleavage. Lung ACE favors the initial Gly-Leu-Met-NH_2 cleavage 3:1, while striatal ACE favors Leu-Met-NH_2 cleavage 1.4:1. Degradation products and ratio of pathways were determined as described in Materials and Methods.

The pattern of SP cleavage by brain ACE differs markedly from the lung enzyme (data not shown). Brain ACE provides a larger proportion of peptides that would arise from an initial removal of the carboxyl terminal dipeptide than is the case with lung ACE.

We confirmed the differential degradation of SP by lung and brain ACE in experiments utilizing [^{3}H]SP (Figure 2). Since the tritium label is located on the proline adjacent to the N-terminal arginine, the only two labeled peptide end products are [^{3}H]Arg-Pro, arising from an initial carboxyl terminal tripeptide cleavage, and [^{3}H]SP 1-5, which results from initial carboxyl terminal dipeptide cleavage. The ratios of [^{3}H]Arg-Pro to [^{3}H]SP 1-5 differ markedly between lung and brain ACE, with substantially more [^{3}H]SP 1-5 formed by brain ACE and more [^{3}H]Arg-Pro formed by lung ACE. From these data one can compute that striatal ACE initially releases the carboxyl terminal dipeptide 1.4 times as often as the tripeptide, while lung ACE releases the carboxyl terminal tripeptide three times more frequently than the dipeptide.

The most striking difference between brain and lung ACE relates to their effects upon SP and SK (Figure 3). The striatal enzyme degrades SP and SK

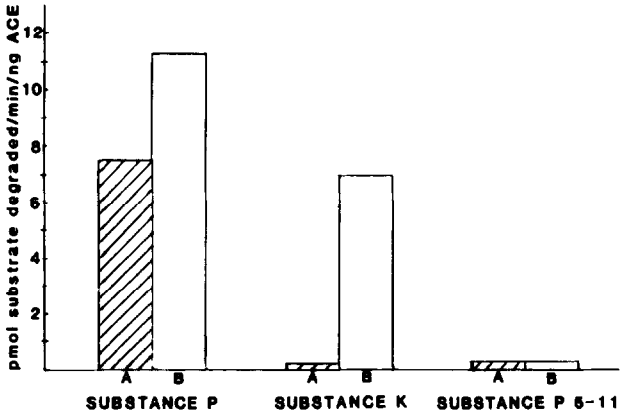

Figure 3 Comparison of Striatal and Lung Cleavages of SP and SK

The degradation of peptides by lung (A) or striatal (B) ACE was monitored by reverse phase HPLC as in Methods. The rate of decrease in UV absorbant material eluting at the position of intact peptide is expressed as pmol substrate degraded/min/ng ACE. The results are the average of three separate determinations which varied by less than 20°/°. The data were determined with less than 50°/° substrate hydrolysis.

to approximately the same extent. By contrast, while SP is an active substrate for lung ACE, SK is not degraded by the lung enzyme even after incubations at 37° for 15 h.

Like other dipeptidyl carboxypeptidases, ACE is thought to recognize primarily the three or four carboxyl terminal amino acids of a peptide (16). Thus, it is striking that the hepapeptide SP 5-11 is not cleaved at all by either lung or striatal ACE despite incubations up to 12 h at 37°.

Discussion

The present results support the possibility that SK and/or SP may be the endogenous substrates for ACE in the striatonigral pathway of the brain. SP has been definitively identified as a major neuropeptide in this pathway (9,10) and indirect evidence suggests that SK also occurs in this neuronal system. In support of a physiological role of ACE in degrading SP, Hanson and Lovenberg (18) found that intraventricular injections of captopril elevate SP levels, while Quik and Emson (10) found that destruction of the striatonigral pathway reduces the degradation of SP by slices of substantia nigra. Others have established that SP can be degraded by ACE via two cleavage patterns similar to what has been found in the present study (14-16). We have quantified the extent of these two pathways and showed marked differences in the preference of the lung and brain enzymes for initial removal of the carboxyl terminal tripeptide or dipeptide respectively. Moreover, we have shown that the lung and brain enzymes differ markedly in their substrate preference with the lung enzyme failing to degrade SK, while the brain enzyme displays similar activity toward SK and SP.

Other peptides are present in the descending striatonigral pathway, including dynorphin and the enkephalins. The enkephalins are degraded by ACE (19), but captopril administration directly into the brain does not alter enkephalin levels, while inhibitors of the endopeptidase enkephalinase do increase enkephalin concentrations (20). The sensitivity of dynorphin to ACE has not been established.

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