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Carboxyl-Terminal Tripeptidyl Hydrolysis of Substance P by Purified Rabbit Lung Angiotensin-Converting Enzyme and the Potentiation of Substance P Activity *in Vivo* By Captopril and MK-422

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SUMMARY

The hydrolysis of substance P is catalyzed by purified rabbit lung angiotensin-converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1). The k_{cat}/K_m for the reaction at 37° is $3.3 \pm 0.3 \times 10^3$ M^{-1} sec^{-1}, which is 60 times less than that which has been reported for the hydrolysis of angiotensin I. The initial site of hydrolysis is the antipenultimate peptide bond, which generates the tripeptide amide (Gly-Leu-Met-NH_2). This hydrolysis is inhibited by the angiotensin-converting enzyme inhibitors captopril, MK-422, and EDTA, and is dependent on the concentration of chloride ion. Both captopril and MK-422 potentiate the substance P-induced stimulation of salivation in rats. Thus, angiotensin-converting enzyme may be one of the enzymes that degrade substance P *in vivo*.

INTRODUCTION

The undecapeptide substance P (Arg[1]-Pro-Lys[3]-Pro-Gln[5]-Gln-Phe[7]-Phe-Gly[9]-Leu-Met[11]-NH_2) has been identified as a potential neurotransmitter/modulator in the central and peripheral nervous systems (1). Pharmacological (2, 3) and receptor studies (3, 4) indicate that substance P fragments smaller than the carboxyl terminal hexapeptide or which do not contain a blocked carboxyl terminal (e.g., substance P free acid) have less than 0.02% or 0.1% of the potency of substance P, respectively. Therefore, proteolytic hydrolysis of a peptide bond in the carboxyl-terminal sequence by a membrane-bound enzyme could be involved in the termination of the activity of substance P at the synaptic cleft.

Several laboratories have investigated the degradation of substance P *in vivo* (5) and *in vitro* (6, 7). Lembeck *et al.* (5) showed that bacitracin, a nonspecific protease inhibitor, potentiated the stimulation of salivation in response to substance P, which was infused simultaneously via the jugular vein. Several membrane-bound enzymes have been identified and purified which catalyze the hydrolysis of one or more peptide bonds in the carboxyl-terminal sequence of substance P (8–10). However, the importance of these enzymes in degrading substance P *in vivo* has not been demonstrated.

Substance P has been reported to inhibit the activity of the dipeptidyl carboxypeptidase, angiotensin-converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1) in striatal homogenates (11), in serum (12), and in a partially purified enzyme preparation (13). Couture and Regoli (7) showed that captopril, an inhibitor of angiotensin-converting enzyme, reduces the rate of inactivation of substance P by rat plasma. In addition, Hanson and Lovenberg (14) demonstrated that intraventricular injection of captopril results in increases in the level of substance P-like immunoreactivity in various central nervous system locations.

Inhibitors of angiotensin-converting enzyme dramatically lower blood pressure in both animal models and humans (15). The presumed mode of action of these drugs is to inhibit the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor (15). Since peripherally administered substance P is a potent vasodilator (16), it was of interest to determine whether captopril and MK-422,[1] an example of a new class of angiotensin-converting enzyme inhibitors (17), could potentiate the activity of substance P *in vivo*. This work demonstrates that the hydrolysis of substance P at the Phe[8]-Gly[9] bond is catalyzed by purified angiotensin-converting enzyme. This work was previously presented in preliminary form.[2]

EXPERIMENTAL PROCEDURES

Synthetic peptides were purchased from Beckman (substance P), Peninsula (substance P fragments, [*N*-methyl-Phe[8], *N*-methyl-Gly[9]]-substance P), Bachem (substance P free acid, substance P methyl ester), Vega Biochemical (leucylmethionine), Serva (leucylmethionine amide), or Chemalog (phenylalanylphenylalanine). All peptides were greater than 95% pure as determined by HPLC. Triethylamine was purchased from Pierce, and HPLC-grade acetonitrile was from Baker.

[1] The abbreviations used are: MK-422, *N*-(1-(*S*)-carboxy-3-phenylpropyl)-L-alanyl-L-proline; HPLC, high-performance liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Me_2SO, dimethyl sulfoxide.

[2] Abstracts of The Endocrine Society Annual Meeting, June 8–10, 1983, San Antonio, Tex.

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Captopril was provided by Dr. Miguel Ondetti, Squibb Institute for Medical Research (Princeton, N. J.).

Salivation assay. The salivation assay was performed as described previously (3). Substance P was dissolved in 0.1 N acetic acid and diluted in 0.15 M NaCl immediately before injection. Sprague-Dawley male rats (body weight 250–350 g) were anesthetized with ether. The peptide was injected via the tail vein or the saphenous vein, and saliva was collected from the buccal cavity with a Pasteur pipette over a period of 2 min. The volume of saliva was measured by pipette. Inhibitors were injected either simultaneously with or 2 min prior to substance P. Three rats were used at each dosage level.

Enzyme purification and assay. Angiotensin-converting enzyme was purified directly to homogeneity from a rabbit lung homogenate by affinity chromatography with *N*-(1-(*S*)-carboxyl-3-phenylpropyl)-L-lysyl-L-proline, as will be described elsewhere.[3] The enzyme obtained from the affinity column was pure by the criterion of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (single band at M_r = 130,000), corresponding to a 1000-fold purification. In support of the purity of this rabbit lung enzyme preparation, the affinity chromatography ligand is extremely specific for angiotensin-converting enzyme: affinity chromatography of the much rarer human plasma enzyme, a source in which it constitutes less than 0.001% of the total protein, affords a 100,000-fold purification of this species in a single step. Thus, we feel confident that the enzyme employed in these studies was very pure. Moreover, repetition of several of these experiments with homogeneous rabbit lung enzyme from another laboratory, kindly provided by Dr. C. Y. Lai at the Roche Institute, gave identical results.

Angiotensin-converting enzyme was assayed with the continuous spectrophotometric substrate furanacryloyl-phenylalanyl-glycyl-glycine, as described by Holmquist *et al.* (18). Standard conditions were 5 nM enzyme and 30 μM substrate in 0.10 M Hepes, 0.30 M KCl, 100 μM $Zn,^{2+}$ and 1 μM bovine serum albumin at pH 7.50 and 25°. Enzyme concentrations were calculated from the first-order rate constants observed at 30 μM substrate and employing the catalytic constants K_m = 300 μM and k_{cat} = 317 sec^{-1} of Holmquist *et al.* (18). Reported K_i values were determined from similar first-order rate constants (k), which were fit by nonlinear regression with a SAS statistical analysis computer program (SAS Institute, Cary, N. C.) to the equation $k = k_0/(1 + (I)/K_i)$; such data give precise K_i values but provide no information about the formal mechanism of inhibition.

HPLC. HPLC was performed using a Varian 5060 liquid chromatograph equipped with a Vista 401 controller and a Beckman 165 two-channel variable wavelength detector. Chromatography was on a Supelcosil C18, 5 μm column (0.4 mm × 25 cm; Supelco, Inc., Bellefonte, Pa.) at 30°. Peptides were eluted at a rate of 1 ml/min with 0.05 M phosphoric acid, buffered to pH 2.5 with triethylamine, and an acetonitrile gradient. Acetonitrile was 5% at 0 min and was increased linearly to 14% at 20 min, 35% at 40 min, and 50% at 50 min. Fifty microliters of the reaction mixture were injected.

Incubation of peptides with angiotensin-converting enzyme. Peptides were incubated with enzyme at 37° in 50 mM Hepes (pH 7.5) containing crystalline bovine serum albumin (300 μg/ml), 0.3 M KCl, 0.1 mM $ZnCl_2$, 5 mM acetic acid, 0.2 mM Tris, 1 μM EDTA, and 10 mM NaCl in a final volume of 0.1 ml. The reaction was terminated by the addition of 2 μl of 0.1 M EDTA and cooling to 0°.

RESULTS

Substance P stimulates saliva secretion in a dose-dependent manner when administered intravenously to rats (19). The time course of this response is shown in Fig. 1. The rate of salivation increases to reach a maximum 20–30 sec after injection, and returns to the basal level after 2 min. The termination of the substance P effect is due to degradation of substance P, since the salivation increases again after a second injection of substance P. When captopril (170 μg/kg, i.v.) is injected 2 min prior to substance P, the rate of salivation increases at all time periods until returning to basal levels by 2.5 min after injection. A summary of the data indicating that the effect of substance P on saliva secretion is potentiated by converting enzyme inhibitors is presented in Table 1. Both MK-422 and captopril increase the volume of saliva secreted in response to substance P at dosages similar to those required to attenuate the hypertensive response elicited by exogenous angiotensin I (15, 17), but have no effect on the basal rate of saliva secretion.

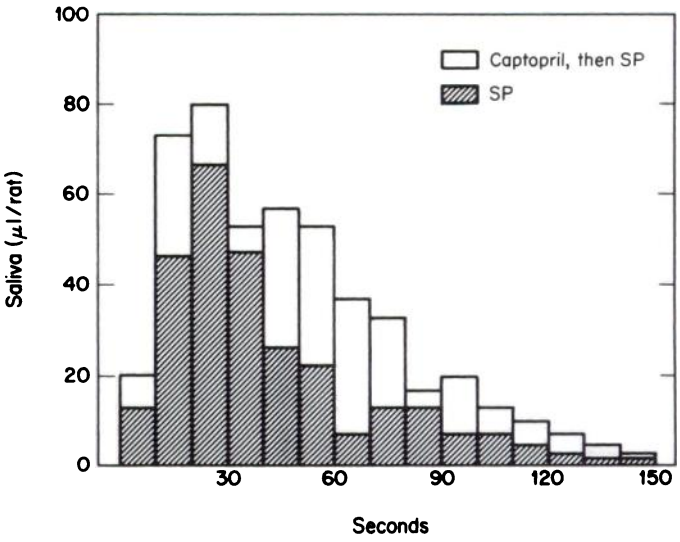

FIG. 1. *Potentiation of substance P-induced salivation by captopril*
Rats were anesthetized with ether and received injections (i.v.) either of saline alone (0.1 ml) or saline plus captopril (170 μg/kg of body weight). Saliva was collected for a 2-min period after the injection and was 0.013 ± 0.005 ml (saline alone) and 0.019 ± 0.019 ml (captopril). Each rat then received an injection of substance P (3 μg/kg of body weight i.v. in 0.1 ml of saline), and saliva was collected every 10 sec immediately after the injection. The results are the average of three rats.

The potentiation of the biological activity of substance P by converting enzyme inhibitors indicates that substance P may be a substrate for converting enzyme. Substance P is separated from all of the potential carboxyl terminal fragments by ion-paired reverse-phase chromatography using an acetonitrile gradient (Fig. 2). This system was utilized to separate intact substance P (0.8 mM) from the products of its reaction with purified angiotensin-converting enzyme (0.34 μM) (Fig. 3).

Substance P, which was greater than 93% pure, eluted at 38.2 min in this system (Fig. 3C). After incubation with converting enzyme, the substance P peak area was decreased, and five product peaks were observed which had retention times of 8.2 min (*I*), 9.2 min (*II*), 12.5 min (*III*), 17.6 min (*IV*), and 30.1 min (*V*) (Fig. 3D). The peak which eluted at 17.6 min (*IV*) was identified as Gly-Leu-Met-NH_2 by its co-elution with standard (Fig. 3B) and by amino acid analysis (data not shown). The 8.2-min peak (*I*) coeluted with Leu-Met-NH_2 but was only 20% of the amount of Gly-Leu-Met-NH_2 observed. No Leu-Met or any other carboxyl terminal sequence of

[3] H. G. Bull, N. A. Thornberry, A. A. Patchett, and E. H. Cordes, manuscript in preparation.

TABLE 1

Potentiation of substance P stimulation of salivation by converting enzyme inhibitors

Substance P and inhibitor were administered (i.v.) to anesthetized Sprague-Dawley rats (250–350 g), and saliva was collected for 2.5 min. In each experiment, the rats used were approximately the same body weight. Inhibitor was injected simultaneously with, or 2 min prior to, substance P.

	Substance P	MK-422	Captopril	Saliva/rat[a]
	μg/rat	*μg/rat*	*μg/rat*	*ml*
Expt. 1	1.4	—	—	0.42 ± 0.08(6)
	1.4	30	—	0.62 ± 0.08(3)**
Expt. 2	0.3	—	—	0.28 ± 0.09(3)
	0.3	3	—	0.21 ± 0.06(3)
	0.3	9	—	0.27 ± 0.05(3)
	0.3	18	—	0.38 ± 0.03(3)
	0.3	30	—	0.46 ± 0.02(3)*
Expt. 3	0.5	—	—	0.24 ± 0.07(3)
	0.5	—	10[b]	0.46 ± 0.03(3)**
Expt. 4	—	—	—	0.01 ± 0.01(3)
	—	—	48[b]	0.03 ± 0.002(3)
	0.9	—	—	0.15 ± 0.04(3)
	0.9	—	1[b]	0.16 ± 0.03(3)
	0.9	—	6[b]	0.32 ± 0.12(3)
	0.9	—	12[b]	0.29 ± 0.03(3)**
	0.9	—	24[b]	0.28 ± 0.08(3)
	0.9	—	48[b]	0.39 ± 0.07(3)**
Expt. 5	0.8	—	—	0.24 ± 0.09(6)
	0.8	—	50[b]	0.39 ± 0.04(3)*
Expt. 6	0.8	—	—	0.245 ± 0.023(3)
	0.8	—	50[b]	0.387 ± 0.034(3)***
Expt. 7	0.8	—	—	0.186 ± 0.092(6)
	0.8	—	50[b]	0.445 ± 0.086(3)***

[a] Values are means ± standard deviation, with number of rats indicated in parentheses. Responses which were significantly different from substance P alone are indicated as follows: * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$.

[b] Inhibitor administered 2 min prior to substance P.

substance P was observed. A major absorbance peak (30.1 min, *V*) co-eluted with one of the two peaks present in a commercially available sample of Phe-Phe (Fig. 3B); this was the only product observed when absorbance was monitored at 254 nm. In addition, when [2-L-Pro-3,4-^{3}H]substance P (10 μCi/ml, 12.5 μCi/μmole; New England Nuclear Corporation) was incubated with converting enzyme, 94% of the radioactive product eluted at 3 min along with the solvent peak (data not shown), indicating that the amino terminal hydrolysis product of the reaction is very polar. Thus, the major site (80%) of initial hydrolysis of substance P catalyzed by converting enzyme is at the Phe^8-Gly^9 bond, and this hydrolysis generates the tripeptide amide (Gly^9-Leu-Met^{11}-NH_2) and substance P(1–8). The latter product is further hydrolyzed by converting enzyme functioning as a dipeptidyl carboxypeptidase. The same fragments are generated by substance P hydrolysis by angiotensin-converting enzyme purified in our laboratories by affinity chromatography or by enzyme provided by Dr. C. Y. Lai which was purified by different methodology as described by Das and Soffer (20). Similar concentrations of these enzyme preparations catalyzed substance P hydrolysis to the same extent.

When substance P free acid was incubated with converting enzyme, Leu-Met (14.4 min) and substance P(1–9) were found (Fig. 3E and F), indicating that the initial hydrolysis is at the Gly^9-Leu^{10} bond as expected. The hydrolysis of substance P in which the carboxyl terminal amide was replaced with a methyl ester (21) is also catalyzed by converting enzyme (Fig. 3G and H). Four of the products co-elute with products of substance P hydrolysis (Fig. 3D), including a peak where Phe-Phe is observed. This suggests that the initial hydrolysis of substance P methyl ester, like the hydrolysis of substance P, is an endopeptidase cleavage which generates the tripeptide methyl ester.

Sandberg *et al.* (22) have reported the synthesis of [*N*-methyl-Phe^8,*N*-methyl-Gly^9]-substance P, and have shown that this analogue is resistant to proteolytic degradation by rat brain membranes. This analogue was incubated with converting enzyme under the same conditions used for substance P in Fig. 3D, and no hydrolysis was observed (data not shown).

The degradation of substance P by purified converting enzyme is inhibited by MK-422, captopril, and EDTA (Table 2). The IC_{50} for MK-422 to inhibit substance P (70 μM) hydrolysis is 0.38 μM. When the chloride ion concentration is reduced to 10 mM, the enzyme retains 62% of its endopeptidase activity (Table 2). In the presence of 0.1 mM chloride ion, which is contributed by the $ZnCl_2$ required to activate the enzyme, the enzyme re-

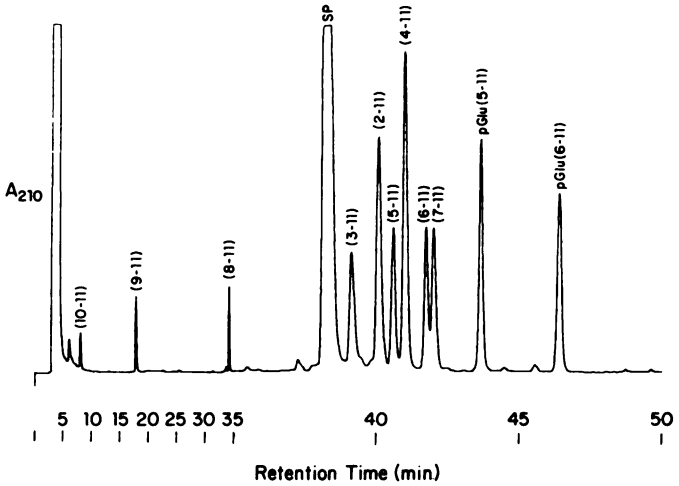

FIG. 2. *Separation of substance P and its carboxyl-terminal fragments by ion-paired reverse-phase chromatography*

A mixture (50 μl) containing substance P (0.6 mM) and substance P(9–11) (0.5 mM) and the eight other potential fragments (0.2 mM) in 60% Me_2SO in 0.04 M acetic acid was injected onto a Supelcosil C18 column and eluted as described under Experimental Procedures. Substance P(6–11) and substance P(5–11) were present as both the free and *N*-cyclized (pGlu) peptides. The large solvent peak and the peak at 6.1 min were due to Me_2SO. Thirty-five minutes after injection, the chart speed was increased 5-fold to ensure clarity.

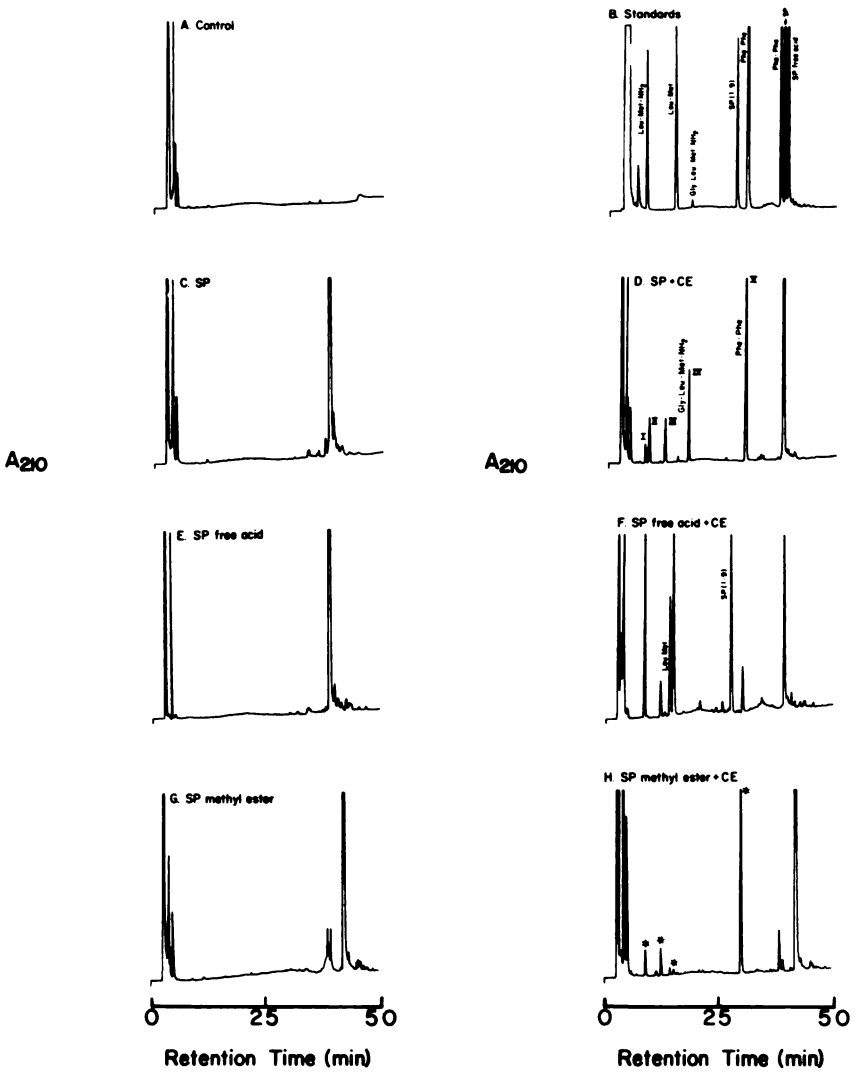

FIG. 3. *Hydrolysis of substance P, substance P free acid, and substance P methyl ester by angiotensin-converting enzyme*

Peptides (0.8 mM) were incubated with enzyme (0.34 μM) at 37° for 3 hr (substance P and substance P methyl ester) or 2 min (substance P free acid) as described under Experimental Procedures. Aliquots (50 μl) were injected onto a Supelcosil C18 column as in Fig. 2. A, converting enzyme only (control); B, a mixture containing Leu-Met (2 mM), Leu-Met-NH_2 (2 mM), Phe-Phe (2 mM), substance P(1–9) (0.2 mM), substance P(9–11) (0.6 mM), substance P (0.3 mM), and substance P free acid (0.3 mM) in 57% Me_2SO in 0.04 M acetic acid (the peak at 6.2 min is due to Me_2SO); C, substance P only; D, substance P and converting enzyme; E, substance P free acid only; F, substance P free acid and converting enzyme; G, substance P methyl ester only; H, substance P methyl ester and converting enzyme (the *asterisks* indicate the products in H that co-elute with products in D.

tains only 35% of its activity. The inhibition of the endopeptidase activity by EDTA and converting enzyme inhibitors, and the chloride dependence of this activity strongly suggest that the activity is due to angiotensin-converting enzyme.

Substance P inhibits the hydrolysis of furanacryloyl-Phe-Gly-Gly by angiotensin-converting enzyme with a K_i of 260 ± 20 μM (Table 3). A double-reciprocal plot of initial velocity versus substrate in the presence and absence of substance P shows that substance P is a competitive inhibitor of furanacryloyl-Phe-Gly-Gly hydrolysis (data not shown). The K_i values for substance P free acid, substance P methyl ester, and [*N*-methyl-Phe^8,*N*-methyl-Gly^9]-substance P are also listed in Table 3. As expected, substance P free acid, which has a free carboxyl-terminal, is an 8-fold better inhibitor than substance P, whereas [*N*-methyl-Phe^8,*N*-methyl-Gly^9]-substance P is only 0.16 as potent as substance P.

In order to measure the rate constant for the catalysis of substance P hydrolysis by angiotensin-converting enzyme, substance P (78 μM) was incubated with converting enzyme (0.34 μM or 0.17 μM) for various time periods before the addition of 2 mM EDTA, and the amount of intact substance P remaining was determined by integration of the peak area after column elution. Substance P was completely degraded after 2 hr (Fig. 4). Since the concentration of substance P was well below its dissociation constant from the enzyme (K_i = 260 μM), its rate of disappearance is expected to be a first-order process. The *solid lines* in Fig. 4 are theoretical fits of the data to a first-order rate equation with rate constants of 6.0 ± 1.5 × 10^{-4} sec^{-1} and 1.1 ± 0.2 × 10^{-3} sec^{-1} at 0.17 and 0.34

TABLE 2

Inhibition of substance P hydrolysis by angiotensin-converting enzyme

Substance P (0.8 mM) was incubated with converting enzyme (0.34 μM) for 4 hr at 37° and chromatographed as described in Fig. 4. Inhibitors were added to the incubation mixture 10 min prior to addition of substrate.

	Substance P degraded
	%
Control	48.5 ± 3.5[a]
+ MK-422 (0.2 mM)	0
+ captopril (0.2 mM)	0
+ EDTA (2 mM)	6
− KCl, + 10 mM NaCl, + 0.1 mM $ZnCl_2$	30
− KCl, −NaCl, + 0.1 mM $ZnCl_2$	17

[a] Mean ± standard deviation for two experiments.

μM enzyme, respectively. The same data are also shown as linear semilog plots in Fig. 5, in which the *solid lines* are those predicted by these rate constants. These rate constants correspond to $k_{cat}/K_m = 3.3 \pm 0.3 \times 10^3$ M^{-1} sec^{-1} for hydrolysis of substance P by angiotensin-converting enzyme. Similar measurements with substance P methyl ester indicated that this analogue is hydrolyzed with $k_{cat}/K_m = 5.6 \times 10^2$ M^{-1} sec^{-1}.

DISCUSSION

Angiotensin-converting enzyme is known to bind peptides which have a free carboxyl-terminal residue preferentially (15). However, the initial hydrolysis of substance P, which contains a carboxyl-terminal amide, at the Phe^8-Gly^9 bond, indicates that highly purified preparations of angiotensin-converting enzyme can also express an endopeptidase activity. This activity was observed previously by Inokuchi and Nagamatsu (23) with peptide substrates containing a penultimate proline residue. Previous work showed that converting enzyme could not hydrolyze the imide bond of a proline residue (15). However, Inokuchi and Nagamatsu demonstrated that Ser-Pro-Phe was the initial hydrolysis product when des-Arg^9-bradykinin ($k_{cat}/K_m = 6.8 \times 10^{-5}$ M^{-1} sec^{-1}) and *N*-benzoyl-Gly-Ser-Pro-Phe ($k_{cat}/K_m = 5.5 \times 10^4$ M^{-1} sec^{-1}) were incubated with purified hog lung or kidney converting enzyme (23).

TABLE 3

Inhibition of angiotensin-converting enzyme-catalyzed hydrolysis of furanacryloyl-Phe-Gly-Gly by substance P and its analogues

The first-order rate constant for the reaction of furanacryloyl-Phe-Gly-Gly (30 μM) and angiotensin-converting enzyme (5 nM) was determined in the presence and absence of inhibitor peptide (16 concentrations, 1 μM to 1 mM) as described under Experimental Procedures.

	K_i[a]
	μM
Substance P	260 ± 20
Substance P free acid	32 ± 4
Substance P methyl ester	290 ± 20
[$MePhe^8$,$MeGly^9$]-Substance P	1600 ± 300

[a] Means ± standard deviation.

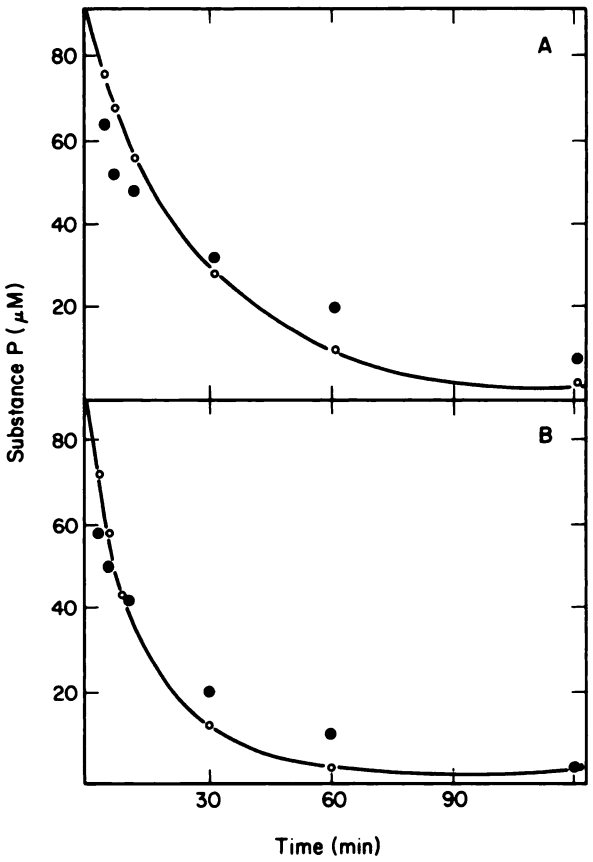

FIG. 4. *Time course of substance P degradation by angiotensin-converting enzyme*

Substance P (0.07 mM) was incubated with angiotensin-converting enzyme (A, 0.17 μM; B, 0.34 μM) at 37° for various times before the addition of EDTA to terminate the reaction. Aliquots (50 μl) were chromatographed as in Fig. 2 to determine the concentration of substance P remaining. The *solid circles* (●) represent the experimentally determined values; the *solid curves* are theoretical and were obtained by nonlinear regression to the expression (substance P) = $(\text{substance P})_0 e^{-kt}$.

It has been suggested (15) that the carboxyl-terminal (R_1) amino acid residue of a peptide interacts ionically with a charged residue of converting enzyme. The carboxyl-terminal peptide bond (R_1—R_2) and the penultimate bond (R_2—R_3) may interact with the enzyme via hydrogen bonding. Catalysis is believed to occur via an electrophilic effect of Zn^{2+} on the R_3 carbonyl and nucleophilic attack by a water molecule at the penultimate peptide bond. The syntheses of active site-directed inhibitors (15, 17) have supported this scheme. Our data and those of Inokuchi and Nagamatsu (23) indicate that, when the carboxyl-terminal residue (R_1) is blocked or when the penultimate residue (R_2) is proline, the active site of converting enzyme will bind the R_3 and R_4 residues of a peptide substrate with low affinity and hydrolyze this bond.

The k_{cat}/K_m for substance P hydrolysis ($3.3 \pm 0.3 \times 10^3$ M^{-1} sec^{-1}) is 60 times less than the k_{cat}/K_m for angiotensin I hydrolysis (1.88×10^5 M^{-1} sec^{-1}) reported by Das and Soffer (20). The catalysis of the hydrolysis of substance P by converting enzyme is relatively insen-

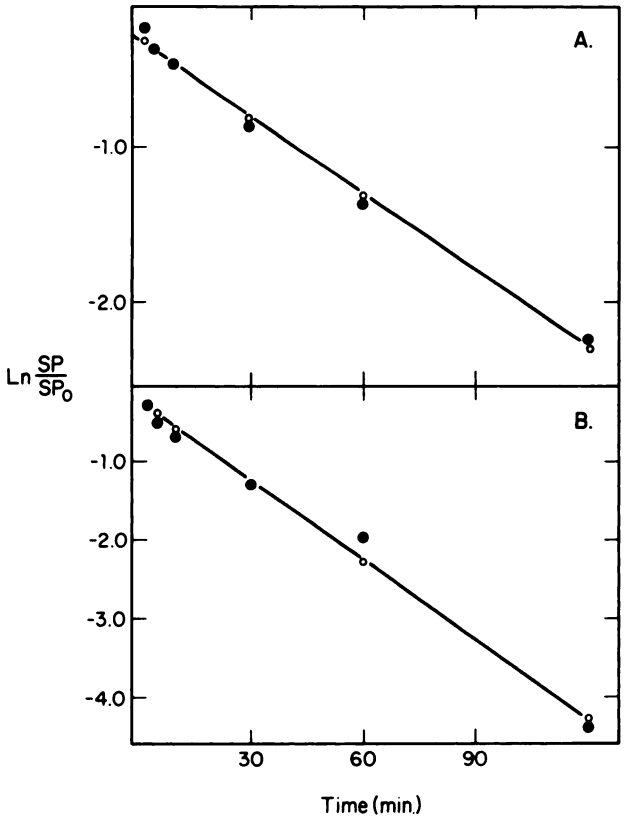

FIG. 5. *Linear semilog plot of the degradation of angiotensin-converting enzyme versus time*

The data in Fig. 4 were replotted as ln (substance P/initial substance P) versus time. The *solid circles* (●) represent experimentally determined values; the *solid line* indicates the theoretical relationship for a reaction with a first-order rate constant of (A) $6.0 \pm 1.5 \times 10^{-4}$ sec^{-1} at 0.17 μM enzyme and (B) $1.1 \pm 0.2 \times 10^{-3}$ sec^{-1} at 0.34 μM enzyme.

sitive to chloride ion. There are many substrates (e.g., bradykinin) for this enzyme whose hydrolysis is equally insensitive to chloride ion (24–26); however, at this time, the reason for this difference is not known. It has been suggested that this low chloride requirement could be physiologically relevant, since optimal hydrolysis of angiotensin I by converting enzyme requires 200 mM NaCl, while hydrolysis of bradykinin and substance P would be maximal under physiological conditions (24).

Previous workers have demonstrated that the hydrolysis of [^{14}C]Hip-His-Leu by rat striatal homogenates (11), partially purified canine lung converting enzyme (13), and human serum (12) is inhibited by substance P at micromolar concentrations. Recently, Komissarova *et al.* (12) demonstrated that Arg-Pro-Lys-Pro [substance P(1–4) and Lys-Pro (substance P(3–4)] inhibit serum enzyme activity as well as substance P, whereas the carboxyl-terminal heptapeptide of substance P does not inhibit at concentrations up to 200 μM. These data suggest that the inhibition of converting enzyme which these workers observed may have been due to amino-terminal fragments of substance P generated by other enzymes in the preparation. Such enzymes are known to occur in serum (27) and in brain tissue (28). Our observation that purified rabbit lung converting enzyme is inhibited by substance P with a K_i of 260 μM, whereas the K_i for substance P free acid is 32 ± 4 μM, is consistent with what is known about the peptide-binding requirements of the enzyme (15).

The catalysis of the hydrolysis of the carboxyl-terminal portion of substance P by other purified membrane proteins has been reported (8–10). Mumford *et al.* (8) showed that substance P hydrolysis is catalyzed by a porcine kidney metalloendopeptidase which appears to be similar to rat striatal "enkephalinase" activity (29). Substance P inhibits the hydrolysis of [^{3}H]leucine enkephalin (20 nM) catalyzed by striatal enkephalinase with an IC_{50} of 3 μM (29). Lee *et al.* (9) purified a membrane-bound substance P-degrading enzyme from human brain which was not inhibited by captopril. The K_m for substance P was 29 μM and the maximal rate of hydrolysis was 27.7 μmoles/hr/mg of protein, giving a k_{cat}/K_m of 9.9×10^3 M^{-1} sec^{-1}. Chubb *et al.* (10) reported the release of carboxyl-terminal methionine from substance P when it was incubated with purified preparations of acetylcholinesterase. This activity co-migrates with the esterase activity on polyacrylamide gel electrophoresis.

The relative importance of converting enzyme and these other peptidases in the termination of substance P action at the synaptosomal membrane is unknown. However, Hanson and Lovenberg (14) showed that intraventricular injection of 1 μg of captopril into rats increased the substance P-like immunoreactivity in the substantia nigra 125% and in the trigeminal nucleus 42%.

The neuroanatomical localization of substance P and substance P-containing nerve tracts has been investigated extensively (1, 30). Defendini *et al.* (31) have used immunohistochemical methods to map the location of converting enzyme in human brain. The enzyme is located on nerve cell membranes at or near the synaptic cleft and in nerve tracts (e.g., striato-nigral) known to contain substance P (1, 30). In addition, Chevillard and Saavedra (32) measured the converting enzyme activity of various areas in the rat brain stem, and, again, the enzyme was present in areas which contained substance P immunoreactive material (1, 30).

These data and our observation that the activity of substance P can be potentiated by both captopril and MK-422 suggest that angiotensin-converting enzyme may play some physiological role in the control of substance P action. The physiological concentration of substance P is probably well below the K_m, so that substance P would be hydrolyzed by a first-order process. The k_{cat}/K_m for hydrolysis of substance P by angiotensin-converting enzyme corresponds to a first-order rate constant of 3×10^{-3} sec^{-1} at 1 μM enzyme, which is equivalent to a half-life of 210 sec. This concentration of angiotensin-converting enzyme is present in lung homogenates.[4] If comparably high levels of angiotensin-converting enzyme are present at the synaptic cleft in certain regions of the central nervous system, then this enzyme may play a significant role in the regulation of substance P levels.

[4] H. G. Bull, unpublished data.

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