In vitro degradation of the C-terminal octapeptide of cholecystokinin by 'enkephalinase A'

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As the C-terminal octapeptide of cholecystokinin represents a putative neurotransmitter in the central nervous system, the membrane-bound enzymes involved in its inactivation were investigated. Two aminopeptidases, involved in the cleavage of enkephalins, and a metalloendopeptidase were identified in extracts of solubilized synaptic membranes. The metalloendopeptidase, which cleaves CCK-8 at the Trp^{30}–Met^{31} bond, appeared to be indistinguishable from 'enkephalinase A_1' on the basis of its chromatographic behaviour, sensitivity to inhibitors and relative affinities for Met- and Leu-enkephalins.

This finding indicates that CCK-8 is inactivated in vitro by the same peptidases as enkephalins.

C-Terminal octapeptide of cholecystokinin *Enkephalin* *Neuropeptidase* *Enkephalinase A* *Metalloendopeptidase* *Solubilized synaptic membrane*

1. INTRODUCTION

The discovery of the gastrointestinal hormone cholecystokinin in the brain of mammals [1–5] and the growing evidence that its C-terminal octapeptide represents a candidate neurotransmitter (or neuromodulator) in the central nervous system led us to search for a specific inactivating enzyme which would destroy the peptide after its discharge in the synaptic cleft.

We described hydrolysis of CCK-8 by soluble [6] and membrane-bound aminopeptidases [7,8] which were previously identified for the cleavage of enkephalins [9–11]. In addition, we observed cleavage of CCK-8 by a metalloendopeptidase from solubilized synaptic membranes whose properties are reported here. On the basis of its chromatographic behaviour, sensitivity to inhibitors and relative affinities for Met- and Leu-enkephalins, this enzyme appeared to be indistinguishable from a peptidase which cleaves enkephalins at the Gly^3–Phe^4 bond and described as 'enkephalinase A_1' [10,12,13].

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Abbreviations: CCK-8, -7, -6, -5, -4, respectively C-terminal octa-, hepta-, hexa-, penta-, tetrapeptide of cholecystokinin; HPLC, high pressure (or performance) liquid chromatography; R_t, retention time; I_{50}, concentration giving half-maximal inhibition

2. MATERIALS AND METHODS

2.1. *Peptides and inhibitors*

Synthetic sulfated CCK-8 (SQ 19,844), Captopril (SQ 14,225) and teprotide (SQ 20,881) were generous gifts from Dr S.J. Lucania (Squibb Institute for Medical Research, Princeton, NJ). Sulfated CCK-7 was supplied by Dr J.S. Morley (ICI, Macclesfield, UK) and bestatin was a gift of Dr H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan).

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2.2. *Separation of the solubilized peptidases from rat synaptic membranes*

Fifteen to twenty Wistar rats were decapitated and whole brains minus cerebellum and brain stem were rapidly dissected. A crude mitochondrial-synaptosomal pellet (P_2) was prepared by a modification of the Whittaker procedure [14]. The membrane pellet obtained after a hypotonic shock was treated with 1 M KCl and extensively washed as previously described [7]. Solubilization was performed according to Gorenstein and Snyder [10]. The solubilized extract was submitted to a DEAE-cellulose chromatography as previously described [7,8].

2.3. *Analysis of CCK-8 cleavage products by HPLC*

CCK-8 or related peptides were incubated for varying times at 37°C with the different chromatographic fractions. The reaction was stopped by boiling and the samples were analyzed by HPLC on μBondapak columns as previously described [7,8] except that the linear acetonitrile gradient was established between 6 and 34%, in a 10 mM ammonium acetate buffer (pH 5.1).

2.4. *Amino-terminal sequence analysis*

Three nmol of sample were analyzed by automated Edman degradation on a modified Beckman sequenator. PTH amino acid derivatives were analyzed by HPLC [8].

3. RESULTS AND DISCUSSION

3.1. *Separation by DEAE-cellulose chromatography of Triton X-100 solubilized CCK-8 cleaving enzymes*

DEAE fractionation of Triton X-100 solubilized synaptic membranes revealed at least five different CCK-8 cleaving enzymes (fig.1). Two aminopeptidases of broad specificity (peaks III and V) gave rise to free tryptophan as the major fluorescent degradation product. These enzymes cleaved the N-terminal tyrosine residue of enkephalins [10,11], the N-terminal tryptophan residue of CCK-4 [7] and hydrolyzed sequentially all the peptide bonds (up to the tryptophan residue) of CCK-8 [8]. CCK-8 was converted into its C-terminal heptapeptide by two proteases (peaks II and IV), one of which corresponded to an amino-

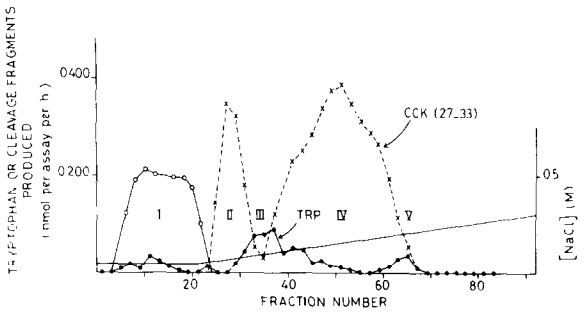

Fig.1. Separation by DEAE-cellulose chromatography of Triton X-100 solubilized CCK-8 cleaving enzymes. Solubilization was performed by incubating the crude synaptic membranes (P_2 pellet) for 30 min at 37°C in 10 vol. 50 mM Tris–HCl buffer (pH 7.7), containing 1% Triton X-100. After centrifugation, the extract was applied to a DEAE-cellulose column (2 × 15 cm) equilibrated in the same buffer containing 0.1% Triton X-100 and eluted using a linear NaCl gradient from 0 to 0.28 M [8]. The fractions were incubated with sulfated CCK-8 (25 μM final concentration) for 5 h at 37°C and the samples were analyzed by reverse-phase HPLC on a μBondapak C_{18} column, using a linear acetonitrile gradient from 6 to 34% [8]. The natural fluorescence of tryptophan or of tryptophan-containing peptides was monitored at 334 nm; the peaks were integrated and quantified using standard fluorescence curves of the appropriate peptide. Free tryptophan (●—●) and the C-terminal heptapeptide of CCK (×—×) were identified on the basis of their retention times as compared with standards. A fluorescent fragment with a R_t = 11.0 min (○—○) was detected in the void volume. Results represent one typical profile out of three.

peptidase A activity (peak II) as previously described [8]. Eluting in the void volume (fig.1: peak I) like 'enkephalinase A_1' [10,12], another CCK-8 cleaving activity gave rise to a tryptophan-containing fragment with a retention time of 11.0 min in our HPLC conditions. This fragment did not elute like standards of CCK-4 (R_t = 14.8 min), CCK-5 (R_t = 15.6 min), CCK-6 (R_t = 17.0 min), unsulfated CCK-7 (R_t = 17.6 min), sulfated CCK-7 (R_t = 15.2 min) or unsulfated CCK-8 (R_t = 15.0 min).

3.2. *Kinetics of cleavage of CCK-8 by peak I and identification of the major fragment produced*

Two fluorescent cleavage products were generated upon incubation of sulfated CCK-8 with peak

I: a major fragment (R_t = 11.0 min) and a minor fragment (R_t = 9.5 min) whose appearance was not linear with time (fig.2). The major cleavage fragment was collected, repurified by HPLC and submitted to N-terminal sequence analysis. The following sequence was found:

$$\begin{array}{l}(SO_3H)\\ \quad|\\ \text{Asp–Tyr–Met–Gly–Trp}\end{array}$$

which corresponds to the N-terminal pentapeptide of CCK-8. This was confirmed by amino acid analysis.

3.3. *Specificity of the enzyme which degraded CCK-8 into its N-terminal pentapeptide*

A dose-related inhibition of the cleavage of CCK-8 by peak I was observed in the presence of Met-enkephalin (I_{50} = 25 μM), (D-Ala^2)Met-enkephalin (I_{50} = 33 μM) and Leu-enkephalin (I_{50} = 120 μM) (fig.3). Furthermore this enzyme gave rise to the Tyr–Gly–Gly fragment when incubated with Leu-enkephalin alone (data not shown). The relative affinities of this CCK-8 cleaving enzyme for Met- and Leu-enkephalin corresponded to the specificity of enkephalinase A described by Snyder [10,12] and others [15]. The cleavage of CCK-8 was also inhibited by other neuropeptides including neurotensin, physalaemin and angiotensin II (data not shown).

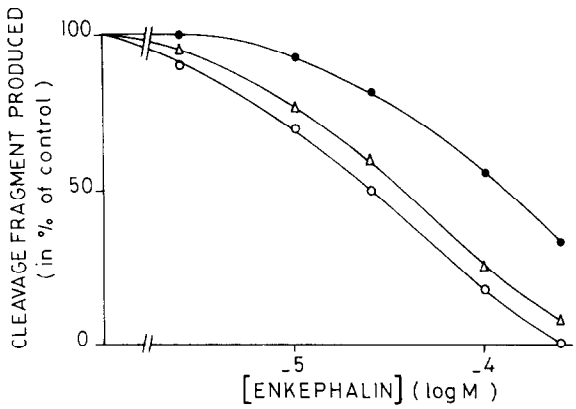

Fig.3. Dose–inhibition curves of enkephalins on the hydrolysis of CCK-8 by peak I. Sulfated CCK-8 (final concentration 25 μM) was incubated for 5 h at 37°C with increasing concentrations of Met-enkephalin (○—○), (D-Ala^2)-Met-enkephalin (△—△) or Leu-enkephalin (●—●) in the presence of 100 μM bestatin. The degradation products were separated by reverse-phase HPLC as described in section 2. The N-terminal fragment of CCK-8 (R_t = 11.0 min) was quantified and expressed in percentage of the amount which appeared in the absence of added enkephalins. Results are the mean of two experiments made in duplicate.

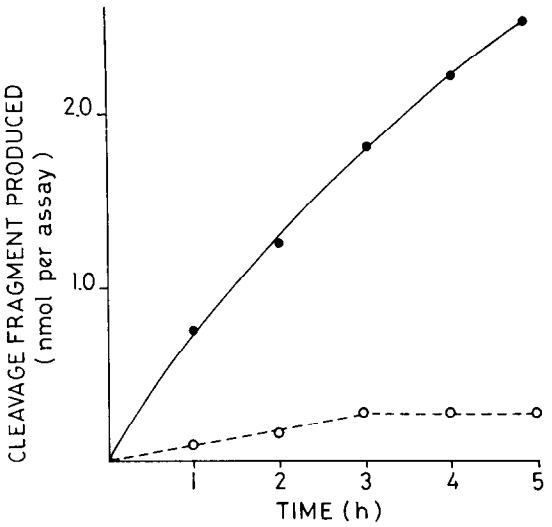

Fig.2. Kinetics of cleavage of CCK-8 by peak I. Incubation of sulfated CCK-8 (final concentration 50 μM) at 37°C with peak I gave rise to a major fluorescent degradation product with a retention time of 11.0 min (●—●) and a minor fragment with a retention time of 9.5 min (○—○). Bestatin (100 μM) was included in the assay medium to inhibit contaminating aminopeptidase activity.

3.4. *Effects of inhibitors on the cleavage of CCK-8 into its N-terminal pentapeptide*

To further clarify the nature of the CCK-8 cleaving enzyme which emerged in the void volume (enkephalinase A or angiotensin-converting enzyme which cleaved also enkephalins at the Gly^3–Phe^4 bond), we evaluated the effect of a variety of protease inhibitors. As shown in table 1, the cleavage of CCK-8 by peak I was completely abolished by 1.5 mM 1,10-phenanthroline indicating that the enzyme involved is a metalloprotease. This cleavage was also markedly inhibited by the dipeptide Tyr–Gly (I_{50} = 60 μM) and 0.2 mM phenobarbital, which are specific enkephalinase A inhibitors, while ACE inhibitors, tested at high concentrations exerted a moderate inhibitory effect.

50 μM *p*-hydroxymercuribenzoate did not signi-

Table 1

Effect of inhibitors on the cleavage of CCK-8 by peak I

Inhibitor	Concentration (mM)	N-terminal fragment (in % of control)
Control	0	100
Tyr–Gly	0.01	100
	0.03	72
	0.1	32
	0.3	7
Phenobarbital	0.2	52
Captopril	1.0	66
SQ 20,881	0.01	86
1,10-Phen-anthroline	1.5	0
PHMB	0.05 (μM)	91
Phosphoramidon	0.01	81
	0.03	43
	0.03	9

Sulfated CCK-8 was incubated at a 25 μM concentration for 5 h at 37°C with peak I, in the presence of 100 μM bestatin and in the absence or presence of various inhibitors. Results are the mean of two experiments made in duplicate

ficantly influence activity. The inhibitory spectrum of the enzyme that cleaved CCK-8 into its N-terminal pentapeptide corresponded to what has been described by many groups for enkephalinase A [10,12,13,15]. Furthermore, the powerful inhibition exerted by phosphoramidon (I_{50} = 23 nM), an inhibitor of thermolysin [16] and membrane-bound metalloendopeptidases [15,17], as well as the nature of the CCK-8 fragment produced (cleavage of the Trp^{30}–Met^{31} bond) indicated that this enzyme is in fact a metalloendopeptidase.

Almenoff et al. [18] and others more recently [19] have raised the question of the identity of a pituitary membrane-bound metalloendopeptidase and of a similar enzyme from kidney membranes [20], with the synaptic membrane-bound enzyme which cleaves enkephalins at the Gly^3–Phe^4 bond.

3.5. *Determination of the kinetic parameters of the metalloendopeptidase for the cleavage of CCK-8*

The production of the N-terminal pentapeptide of CCK-8 by the metalloendopeptidase obeyed Michaelis–Menten kinetics and a K_m of 66 μM was calculated for the cleavage of sulfated CCK-8. K_i values of Met-enkephalin and Leu-enkephalin were determined from the I_{50} (fig.3) assuming a competitive inhibition of CCK-8 cleavage. Values of 17 μM and 85 μM were found respectively, which are in agreement with the K_i of 10 μM reported for Met-enkephalin hydrolysis by solubilized enkephalinase A [10] and with the K_m of 78–79 μM determined by Rush and Hersh [21] for Leu-enkephalin cleavage by enkephalinase A_1. The discrepancy observed between these values and those determined with synaptic membranes may result from the assay conditions (solubilization by Triton X-100, alkaline medium).

The V_{max} of the solubilized metalloendopeptidase for sulfated CCK-8 cleavage was 44.0 pmol/min/assay as compared to values of 27.4 pmol/min/assay for Met-enkephalin and 62.5 pmol/min/assay for Leu-enkephalin. These results indicate that, in vitro, the affinity of enkephalinase A and its rate of cleavage of CCK-8, are of the same order of magnitude as the kinetic parameters determined for Met- and Leu-enkephalins degradation.

3.6. *Influence of the N-terminal region of CCK-8 on the rate of hydrolysis of the Trp^{30}–Met^{31} bond by the metalloendopeptidase*

Figure 4 represents the kinetics of appearance of the N-terminal fragments of sulfated and unsulfated CCK-7 and -8. It is obvious that the presence of the negatively charged sulfate group enhanced the rate of cleavage of the peptide as compared to its unsulfated form. The presence of the N-terminal aspartic residue in the octapeptide increased also the rate of cleavage of the Trp–Met bond, as seen from the comparison between sulfated CCK-7 and -8 as well as between unsulfated CCK-7 and -8.

In conclusion, it appears that the C-terminal octapeptide of cholecystokinin is inactivated by three synaptic membrane-bound enzymes also involved in the cleavage of enkephalins: two aminopeptidases ([8] and fig.1) and a metalloendopeptidase previously described as 'enkephalinase A_1' [10]. A dipeptidyl-carboxypeptidase activity as originally described in [22] was not detected for the cleavage of CCK-8.

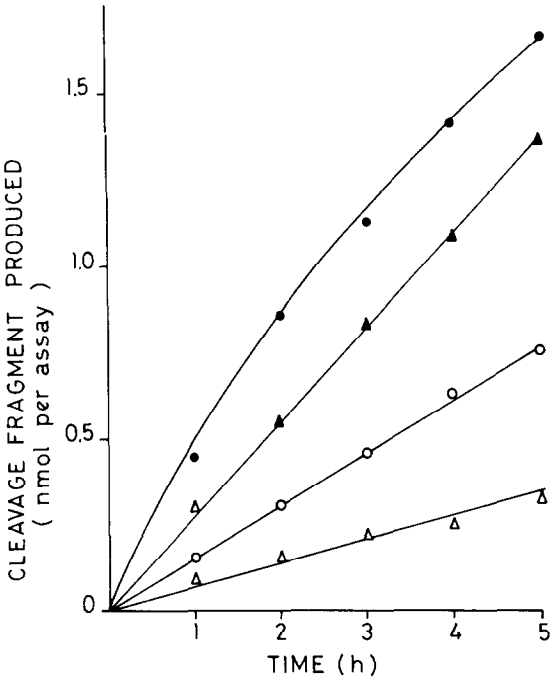

Fig.4. Kinetics of cleavage of CCK-8 related peptides by peak I. Sulfated CCK-8 (●—●), unsulfated CCK-8 (○—○), sulfated CCK-7 (▲—▲) or unsulfated CCK-7 (△—△) were incubated at a 25 μM final concentration for varying times at 37°C in the presence of 100 μM bestatin. Their N-terminal fragments were quantified. Results are the mean of two experiments made in duplicate.

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REFERENCES

[1] Vanderhaeghen, J.J., Signeau, J.C. and Gepts, W. (1975) Nature 257, 604–605.
[2] Dockray, G.J. (1976) Nature 264, 568–570.
[3] Muller, J.E., Straus, E. and Yalow, R.S. (1977) Proc. Natl. Acad. Sci. USA 74, 3035–3037.
[4] Rehfeld, J.F. (1978) J. Biol. Chem. 253, 4022–4030.
[5] Robberecht, P., Deschodt-Lanckman, M. and Vanderhaeghen, J.J. (1978) Proc. Natl. Acad. Sci. USA 75, 524–528.
[6] Deschodt-Lanckman, M., Bui, N.D., Noyer, M. and Christophe, J. (1981) Reg. Peptides 2, 15–30.
[7] Deschodt-Lanckman, M. and Bui, N.D. (1981) Peptides 2, suppl. 2, 113–118.
[8] Deschodt-Lanckman, M., Bui, N.D., Koulischer, D., Paroutaud, P. and Strosberg, A.D., submitted.
[9] Meek, J.L., Yang, H.-Y.T. and Costa, E. (1977) Neuropharmacology 16, 151–154.
[10] Gorenstein, C. and Snyder, S.H. (1979) Life Sci. 25, 2065–2070.
[11] Hersh, L.B. (1981) Biochemistry 20, 2345–2350.
[12] Gorenstein, C. and Snyder, S.H. (1980) Proc. R. Soc. London B 210, 123–132.
[13] Vogel, Z. and Altstein, M. (1980) Prog. Biochem. Pharmacol. 16, 49–59.
[14] Whittaker, V.P. (1959) Biochem. J. 72, 694–706.
[15] Hudgin, R.L., Charleson, S.E., Zimmerman, M., Mumford, R. and Wood, P.L. (1981) Biochem. Biophys. Res. Commun. 29, 2593–2601.
[16] Komiyama, T., Suda, H., Aoyagi, T., Takeuchi, T., Umezawa, H., Fujimoto, K. and Umewazu, S. (1975) Arch. Biochem. Biophys. 171, 727–731.
[17] Fulcher, I.S., Matsas, R., Turner, A.J. and Kenny, A.J. (1982) Biochem. J. 203, 519–522.
[18] Almenoff, J., Wilk, S. and Orlowski, M. (1981) Biochem. Biophys. Res. Commun. 102, 206–214.
[19] Malfroy, B. and Schwartz, J.-C. (1982) Biochem. Biophys. Res. Commun. 106, 276–285.
[20] Mumford, R.A., Pierzchala, P.A., Strauss, A.W. and Zimmerman, M. (1981) Proc. Natl. Acad. Sci. USA 78, 6623–6627.
[21] Rush, R.J. and Hersh, L.B. (1982) Life Sci. 31, 445–451.
[22] Malfroy, B., Swerts, J.P., Guyon, A., Roques, B.P. and Schwartz, J.-C. (1978) Nature 276, 523–526.