Endopeptidase 3.4.24.11 converts *N*-1-(*R,S*)carboxy-3-phenylpropyl-Ala-Ala-Phe-*p*-carboxyanilide into a potent inhibitor of angiotensin-converting enzyme

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It was reported recently that *N*-1-(*R,S*)carboxy-3-phenylpropyl-Ala-Ala-Phe-*p*-carboxyanilide (CPP-A-A-F-pAB), an inhibitor of endopeptidase 3.4.24.15 (E-24.15), also inhibits angiotensin-converting enzyme (ACE) from rabbit lung. We have found that this compound is without effect on ACE purified from pig kidney, at a concentration some 1000-fold greater than the K_i reported for inhibition of the enzyme from lung. However, preincubation of CPP-A-A-F-pAB with neutral endopeptidase 3.4.24.11 (E-24.11) does result in potent inhibitory effects on

ACE. We have shown this to be due to formation of a fragment, CPP-A-A, the structure of which is closely related to ACE inhibitors such as enalaprilat. CPP-A-A was found to be a potent inhibitor of pig ACE. Under the conditions used it had an IC_{50} value of 1.6×10^{-8} M, compared with the value obtained for captopril of 7.5×10^{-10} M. These results have important implications for studies of E-24.15 when using CPP-A-A-F-pAB *in vivo* or in crude tissue extracts where E-24.11 might also be present.

INTRODUCTION

During studies of the proteolytic cleavage by a membrane preparation from rat brain of fragments of the 42-residue β /A4 amyloid peptide associated with Alzheimer's disease (T. Yamamoto, D. Allsop, D. M. Walsh and C. H. Williams, unpublished work), we had cause to examine the effects of *N*-1-(*R,S*)carboxy-3-phenylpropyl-Ala-Ala-Phe-*p*-carboxyanilide (CPP-A-A-F-pAB), a specific inhibitor of endopeptidase 3.4.24.15 (E-24.15) developed by Orlowski et al. (1988). Some of the results suggested that phosphoramidon, an inhibitor of neutral endopeptidase 3.4.24.11 (E-24.11), had effects on the inhibitory properties of CPP-A-A-F-pAB. We decided to investigate possible reasons for this, and now report the findings.

E-24.15 is found in several mammalian tissues, including brain (Orlowski et al., 1983; Acker et al., 1987; Millican et al., 1991), testes (Orlowski et al., 1989; Pierotti et al., 1990) and muscle (Tisljar and Barrett, 1990), but its physiological role is not clear. It exists in both membrane-bound and soluble forms. A related enzyme, endopeptidase 3.4.24.16, is believed to be involved in the metabolism of neurotensin (Checler et al., 1985, 1986; Barelli et al., 1988a,b; Millican et al., 1991) and is also inhibited by CPP-A-A-F-pAB. This inhibitor has been shown to have anti-hypertensive effects in the rat (Genden and Molineaux, 1991), raising the question of whether E-24.15 is involved in metabolism of peptides that have effects on vasoconstriction or electrolyte and water balance (Chappell et al., 1991). Recently, Chappell et al. (1992) have reported that CPP-A-A-F-pAB is also a good competitive inhibitor of angiotensin-converting enzyme (ACE) (K_i 1.9×10^{-7} M), and have suggested that it may be the inhibition of ACE and not of E-24.15 that is responsible for the observed vasoactive effects of the compound. This is obviously of importance, since there is considerable interest in inhibitors of ACE as anti-hypertensive agents, and in the physiological role of E-24.15. Our results show that CPP-A-A-F-pAB does not inhibit

ACE unless E-24.11 is present. Inhibition of ACE under these conditions appears to be due to formation of the cleavage product CPP-A-A, produced by the action of E-24.11 on the Ala-Phe bond of the E-24.15 inhibitor.

MATERIALS AND METHODS

Captopril, phosphoramidon, [leucine]enkephalin, Ala-Ala, *N*-[3-(2-furyl)acryloyl]-Phe-Gly-Gly (FA-F-G-G) and rabbit lung ACE were from Sigma Chemical Co., Poole, Dorset, U.K. CPP-A-A-F-pAB was from Novabiochem, Switzerland. ACE and E-24.11, both purified from pig kidney, were gifts from the Membrane Peptidase Research Group (Leeds, U.K.) (Professor A. J. Turner and Dr. A. J. Kenny). Ethyl 2-oxo-4-phenylbutyrate and sodium cyanoborohydride were from Aldrich Chemical Co., Gillingham, Dorset, U.K.

All enzyme assays except the spectrophotometric assay of Holmquist et al. (1979) (see below) were carried out in duplicate at 37 °C in 0.1 M Tris/HCl buffer, pH 8.2, containing NaCl (300 mM) and 1% (w/v) Triton X-100. Assay of enkephalin cleavage by ACE from pig kidney was carried out as follows, in a total volume of 250 μ l. ACE (0.2 μ g/ml protein in the final assay mixture) and [leucine]enkephalin (1 mM) were incubated with and without the inhibitors captopril (10 μ M) and CPP-A-A-F-pAB (200 μ M) for 1 h at 37 °C. Reaction tubes were placed in a boiling-water bath for 5 min and then centrifuged at 17000 g for 10 min. Samples of the supernatants were analysed by reverse-phase h.p.l.c. (see below). To test the effects of E-24.11, CPP-A-A-F-pAB (200 μ M) was incubated with a sample of this enzyme (0.025 μ g of protein) under the conditions described above. After 30 min, the E-24.11 was inactivated by placing the samples in a boiling-water bath for 5 min. They were then cooled, ACE (0.05 μ g) and [leucine]enkephalin (1 mM) were added and the incubation was continued for a further 1 h at 37 °C. Reactions were terminated by boiling as above. Tubes were centrifuged at

Figure 1 H.p.l.c. profiles showing that E-24.11 is required to produce inhibition of pig kidney ACE by CPP-A-A-F-pAB

(a) Result of 1 h incubation of [leucine]enkephalin with ACE; (b) as (a), but in the presence of captopril ($10 \mu\text{M}$). In (c) CPP-A-A-F-pAB was preincubated (30 min) with E-24.11 before addition of ACE and [leucine]enkephalin, thereafter as in (a); (d), as (c), but the preincubation mixture did not contain E-24.11. Peaks in each case are [leucine]enkephalin (1), Phe-Leu (2), Tyr-Gly-Gly (3), the *R* and *S* enantiomers of CPP-A-A-F-pAB (4), and a cleavage product (5). See the Materials and methods section for further experimental details.

17000 *g* for 10 min, and samples of the supernatants were analysed immediately or stored at -20°C pending h.p.l.c. analysis. Two sets of control experiments were carried out in a similar manner, i.e. for successive 30 min and 60 min incubation times. In one of these sets of experiments the E-24.11 was omitted, and in the other both ACE and [leucine]enkephalin were omitted.

To test the effects of phosphoramidon on metabolism of CPP-A-A-F-pAB, the former ($10 \mu\text{M}$) was incubated with E-24.11 ($0.025 \mu\text{g}$) and CPP-A-A-F-pAB ($200 \mu\text{M}$) for 1 h at 37°C . Samples were prepared for h.p.l.c. as described above. ACE from rabbit lung ($0.25 \mu\text{g}$ of protein) was incubated for 30 min at 37°C in presence of CPP-A-A-F-pAB ($200 \mu\text{M}$), before analysis by h.p.l.c.

H.p.l.c. analysis

Samples ($50 \mu\text{l}$) of the supernatants were analysed by reverse-phase h.p.l.c. on a C_{18} column (Bio-Rad RP-318) using a Shimadzu system. Elution solvents were 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile, with a gradient of 0–80% acetonitrile over 20 min, and a flow rate of 1.5 ml/min. Enkephalin metabolites (YGG and FL) were identified by comparison with authentic standards. The inhibitor CPP-A-A-F-pAB was eluted as a pair of closely spaced peaks of equal height; these were the *R*- and *S*-enantiomers (Novabiochem, personal communication; see also Orłowski et al., 1988). All h.p.l.c. profiles shown were recorded on a Shimadzu C-R4AX Chromatopac system at attenuation level 9.

Preparation and assessment of CPP-A-A as an inhibitor of ACE

CPP-A-A was prepared by reductive alkylation of Ala-Ala with 2-oxo-4-phenylbutyric acid as described by Orłowski et al. (1988) for the analogous preparation of CPP-A-A-F-pAB. The method is essentially the same as that used by Patchett et al. (1980) for preparation of enalaprilat and several of its analogues. The material was purified as described for enalaprilat by those authors. Fast-atom-bombardment m.s. of the product gave a molecular-ion peak of 323, equivalent to $M+1$ for CPP-A-A.

The inhibitory effect of CPP-A-A against pig ACE was compared with that of captopril as follows. Different concentrations of either CPP-A-A or captopril were incubated for 10 min with ACE at pH 7.5 and 30°C before measuring enzyme activity by the spectrophotometric method of Holmquist et al. (1979), with the substrate FA-F-G-G (0.1 mM) at 328 nm. Initial rates were compared with that obtained in the absence of inhibitor, and results were plotted as residual enzyme activity against concentration of inhibitor.

RESULTS

Figure 1(a) shows the cleavage of [leucine]enkephalin by ACE from pig kidney. This cleavage was completely inhibited by captopril ($10 \mu\text{M}$) (Figure 1b). Figures 1(c) and 1(d) demonstrate the effects of CPP-A-A-F-pAB on the inhibition of ACE with and without prior incubation of the inhibitor with E-24.11, respectively. Figure 1(c) also shows the presence of a new peak with a retention time of 10.4 min (peak 5), which does not appear in Figure 1(d) and was found to co-elute with CPP-A-A (results not shown). This peak was also seen when E-24.11 was incubated

Figure 2 Metabolism of CPP-A-A-F-pAB by E-24.11

(a) H.p.l.c. profile after 1 h incubation of E-24.15 inhibitor and enzyme; (b), as (a), but in the presence of phosphoramidon ($10 \mu\text{M}$). The pair of peaks numbered 1 are the *R* and *S* enantiomers of CPP-A-A-F-pAB. It can be seen that a cleavage product identified as peak 2 in (a) is greatly diminished in (b). Peak 3 is phosphoramidon.

Figure 3 Inhibition of pig kidney ACE by CPP-A-A or captopril

Pig ACE ($5 \mu\text{l}$) was preincubated for 10 min at 30°C in 0.95 ml of 50 mM Tris/HCl buffer, pH 7.5, containing 300 mM NaCl, with indicated concentrations of either CPP-A-A ($\circ$) or captopril ($\bullet$) before assaying for enzyme activity at 328 nm by the method of Holmquist et al. (1979). The total assay volume was 1 ml . Points are the averages of duplicate determinations (mean difference indicated by vertical bars) and refer to the initial rates of hydrolysis. The initial reaction rate for the uninhibited enzyme was 25.7 nmol/min . See the Results section for IC_{50} values.

with CPP-A-A-F-pAB, but was greatly decreased in the presence of phosphoramidon (Figure 2).

The results for inhibition of pig ACE by CPP-A-A are shown in Figure 3. Under the conditions used, this substance was found

to be an excellent inhibitor ($\text{IC}_{50} 1.6 \times 10^{-8} \text{ M}$), though not as potent as captopril ($\text{IC}_{50} 7.5 \times 10^{-10} \text{ M}$).

DISCUSSION

The failure of CPP-A-A-F-pAB to inhibit ACE was unexpected, in view of the report by Chappell et al. (1992), that it inhibits the enzyme from rabbit lung in a competitive manner, with a K_i of $1.9 \times 10^{-7} \text{ M}$, especially since the concentration of inhibitor used was approx. 1000 times this value. When CPP-A-A-F-pAB was preincubated with E-24.11 before adding ACE, complete inhibition of the cleavage of [leucine]enkephalin by the latter was observed, suggesting that a metabolite of CPP-A-A-F-pAB may be the inhibitory agent. This is supported by the h.p.l.c. profiles in Figures 1(c) and 2. The findings are in keeping with the observation made by Orlowski et al. (1988) that E-24.15 inhibitors of this type are also weakly inhibitory towards E-24.11, on account of their acting as poor substrates for that enzyme. Similarly, they are consistent with the report that CPP-A-A-F-pAB prolongs the vasodilatory effects of bradykinin (Genden and Molineaux, 1991), since this can be explained by its prior conversion into an ACE inhibitor by E-24.11, an endopeptidase widely distributed in mammalian tissues (Turner, 1987). Such an action would not only impair the production of the vasoconstricting agent angiotensin II, but might also slow down the metabolism of bradykinin, a peptide known to be a good substrate for ACE. A further piece of supporting evidence for this explanation is our observation that the inhibition by CPP-A-A-F-pAB of a captopril-sensitive enzyme (presumably ACE) in rat brain membranes is prevented by phosphoramidon (T. Yamamoto, D. Allsop, D. M. Walsh and C. H. Williams, unpublished work). Thus ACE from pig kidney and rat brain behave similarly, and a possible explanation for the behaviour of the enzyme from lung is that it contains some E-24.11-like activity. This seems more likely than the alternative which is that the lung enzyme is uniquely sensitive to unmetabolized CPP-A-A-F-pAB. It is worth noting that this compound is similar in structure to succinyl-Ala-Ala-Phe-(4-methyl)-7-coumarylamide, a fluorogenic substrate that is cleaved at the Ala-Phe bond by E-24.11 (Mumford et al., 1981; J. Brownlees and C. H. Williams, unpublished work), in keeping with the known specificity of this enzyme, which cleaves peptide bonds on the amino side of hydrophobic amino acids (Turner, 1987). Similarly, the only plausible cleavage site for E-24.11 in CPP-A-A-F-pAB is at the Ala-Phe bond. CPP-A-A, the most likely metabolite, belongs to a class of compounds exemplified by enalaprilat, *N*-1-(3-carboxy)-phenylpropyl-Ala-Pro (CPP-A-P), which is a slow tight-binding inhibitor of ACE, with a K_i for this process of about $2 \times 10^{-10} \text{ M}$ (Patchett and Cordes, 1985). Our finding that CPP-A-A is indeed a potent inhibitor of ACE and has a retention time on reverse-phase h.p.l.c. the same as for the proposed fragment derived from CPP-A-A-F-pAB provides strong confirmatory evidence for our conclusions. Clearly, although CPP-A-A-F-pAB and analogues are excellent inhibitors of E-24.15, their use *in vivo*, or *in vitro* with tissue preparations containing E-24.11, may lead to misinterpretation of results.

The observations of Chappell et al. (1992) are consonant with our findings if it is assumed that their sample of ACE contained some E-24.11, an enzyme known to be present in pulmonary tissue, but with a distribution different from that of ACE (Turner, 1987). They report that in an experiment to test whether CPP-A-A-F-pAB ($50 \mu\text{M}$) can act as a substrate for ACE, no metabolites were detected by reverse-phase h.p.l.c., which also appears to exclude an action of contaminant E-24.11. However, we have found that rabbit lung ACE from the same supplier (Sigma,

A 6778) when incubated with CPP-A-A-F-pAB does produce a new peak, with a retention time the same as that seen in Figures 1(c) and 2(a). This peak was exceedingly small, but its presence, and the fact that it co-elutes with CPP-A-A, provides strong evidence that E-24.11 is a minor contaminant, particularly since the peak was not seen when phosphoramidon was added to the lung ACE (results not shown). This confirms our findings with the purified E-24.11. The failure of Chappell et al. (1992) to detect such metabolism may be because of the small amount of the fragment produced. Indeed, the potency of CPP-A-A, only 20-fold less than captopril in our assay, is such that turnover of only 1 or 2 % of CPP-A-A-F-pAB, at an initial concentration of 50 μ M, would yield a sufficient concentration of CPP-A-A to produce substantial or total inhibition of ACE, depending on the efficiency and concentration of competing substrate.

Although these findings cannot totally exclude the possibility that E-24.15 may be involved in the metabolism *in vivo* of vasoactive peptides such as bradykinin, they do suggest that the evidence on which this proposition is based must be re-appraised.

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REFERENCES

- Acker, G. R., Molineaux, C. and Orlowski, M. (1987) *J. Neurochem.* **48**, 284–292
- Barelli, H., Girard, F., St. Pierre, S., Kitabgi, P., Vincent, J.-P. and Checler, F. (1988a) *Neurochem. Int.* **12**, 351–359
- Barelli, H., Vincent, J.-P. and Checler, F. (1988b) *Eur. J. Biochem.* **175**, 481–489
- Chappell, M. C., Jaiswal, R., Brosnihan, K. B. and Ferrario, C. M. (1991) *Hypertension* **18**, 385–386
- Chappell, M. C., Welches, W. R., Brosnihan, K. B. and Ferrario, C. M. (1992) *Peptides* **13**, 943–946
- Checler, F., Vincent, J.-P. and Kitabgi, P. (1985) *J. Neurochem.* **45**, 1509–1513
- Checler, F., Vincent, J.-P. and Kitabgi, P. (1986) *J. Biol. Chem.* **261**, 11274–11281
- Genden, E. M. and Molineaux, C. J. (1991) *Hypertension* **18**, 360–365
- Holmquist, B., Bunning, P. and Riordan, J. F. (1979) *Anal. Biochem.* **95**, 540–548
- Mumford, R. A., Pierzchala, P. A., Strauss, A. W. and Zimmerman, M. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6623–6627
- Millican, P. E., Kenny, A. J. and Turner, A. J. (1991) *Biochem. J.* **276**, 583–591
- Orlowski, M., Michaud, C. and Chu, T. G. (1983) *Eur. J. Biochem.* **135**, 81–88
- Orlowski, M., Michaud, C. and Molineaux, C. J. (1988) *Biochemistry* **27**, 597–602
- Orlowski, M., Reznik, S., Ayala, J. and Pierotti, A. R. (1989) *Biochem. J.* **261**, 951–958
- Patchett, A. A. and Cordes, E. H. (1985) *Adv. Enzymol.* **57**, 1–84
- Patchett, A. A., Harris, E., Tristram, E. W., Wyvratt, M. J., Wu, M. T., Taub, D., Peterson, E. R., Ikeler, T. J., ten Broeke, J., Payne, L. G., Ondeyka, D. L., Thorsett, E. D., Greenlee, W. J., Lohr, N. S., Hoffsommer, R. D., Joshua, H., Ruyle, W. V., Rothrock, J. W., Aster, S. D., Maycock, A. L., Robinson, F. M., Hirschmann, R., Sweet, S. C., Ulm, E. H., Gross, D. M., Vassil, T. C. and Stone, C. A. (1980) *Nature (London)* **288**, 280–283
- Pierotti, A., Dong, K.-W., Glucksman, M. J., Orlowski, M. and Roberts, J. L. (1990) *Biochemistry* **29**, 10323–10329
- Tislar, U. and Barrett, A. J. (1990) *Biochem. J.* **267**, 531–533
- Turner, A. J. (1987) in *Neuropeptides and their Peptidases* (Turner, A. J., ed.), pp. 183–201, Ellis Horwood, Chichester