Role of angiotensin converting enzyme in the vascular effects of an endopeptidase 24.15 inhibitor

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1 We investigated the role of angiotensin converting enzyme (ACE) in the cardiovascular effects of N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (cFP), a peptidase inhibitor selective for metalloendopeptidase (EP) E.C. 3.4.24.15.

2 In conscious rabbits, cFP (5 mg kg⁻¹, i.v.) markedly slowed the degradation of [³H]-bradykinin, potentiated the depressor response to right atrial administration of bradykinin (10–1000 ng kg⁻¹), and inhibited the pressor response to right atrial angiotensin I (10–100 ng kg⁻¹). In each of these respects, the effects of cFP were indistinguishable from those of the ACE inhibitor, captopril (0.5 mg plus 10 mg kg⁻¹ h⁻¹ i.v.). Furthermore, the effects of combined administration of cFP and captopril were indistinguishable from those of captopril alone.

3 In experimentally naive anaesthetized rats, cFP administration (9.3 mg kg⁻¹, i.v.) was followed by a moderate but sustained fall in arterial pressure of 13 mmHg. However, in rats pretreated with bradykinin (50 µg kg⁻¹) a more pronounced fall of 30 mmHg was observed. Captopril (5 mg kg⁻¹) had similar hypotensive effects to those of cFP, and cFP had no effect when it was administered after captopril.

4 CFP displaced the binding of [¹²⁵I]-351A (the *p*-hydroxybenzamidine derivative of lisinopril) from preparations of rat plasma ACE and solubilized lung membrane ACE (K_D = 1.2 and 0.14 µM respectively), and inhibited rat plasma ACE activity (K_I = 2.4 µM). Addition of phosphoramidon (10 µM), an inhibitor of a range of metalloendopeptidases, including neutral endopeptidase (E.C.3.4.24.11), markedly reduced the potency of cFP in these systems.

5 Taken together these findings suggest that the actions of cFP *in vivo* are attributable to inhibition of ACE rather than EP 24.15. Given that cFP is a poor inhibitor of ACE in the presence of phosphoramidon *in vitro*, it is likely that cFP is cleaved by a phosphoramidon-sensitive metallopeptidase *in vivo* to liberate N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala, a potent ACE inhibitor.

Keywords: Angiotensin converting enzyme; blood-pressure-physiology; bradykinin; metalloendopeptidase 24.15

Introduction

Recently there has been renewed interest in the role that metalloendopeptidases play in blood pressure control, and thus their potential as therapeutic agents (Carretero & Scicli, 1991). Much of this interest has focussed on endopeptidase E.C.3.4.24.15 (EP 24.15), first identified in soluble fractions of brain homogenates by Orlowski *et al.* (1983). Although this enzyme is widely distributed in animal tissue, it is found predominantly in the brain, pituitary and testis, with lower levels in other tissues, such as liver, kidney, lung and spleen (Chu & Orlowski, 1985). In the brain, endopeptidase 24.15 exists in two forms, the predominant soluble form constituting about 80% of the total activity and a membrane-bound form accounting for the remainder of activity (Acker *et al.*, 1987). Both the soluble and membrane-bound forms of the enzyme convert larger opioid peptides, such as dynorphin^{1–8}, α - and β -neoendorphin and Met-enkephalin-Arg-Gly-Leu into bioactive pentapeptides (Acker *et al.*, 1987; Orlowski *et al.*, 1988; 1989). The enzyme also has high affinity for several other peptides such as bradykinin, neurotensin and angiotensin II, which it converts to inactive forms (Orlowski *et al.*, 1983; Chu & Orlowski, 1985).

The design and synthesis of selective EP 24.15 inhibitors has allowed investigation of the possible involvement of EP 24.15 in the regulation of physiological processes. Orlowski

et al. (1988) demonstrated that peptides containing a free N-(carboxymethyl) group, capable of interacting with the active site zinc ion of EP 24.15, act as transition state inhibitors of this enzyme. The most potent and selective of these EP 24.15 inhibitors is N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (cFP), with a reported inhibitory constant (K_i) of 16 nM (Chu & Orlowski, 1984; Orlowski *et al.*, 1988).

The impetus for the present study results from a report showing that in anaesthetized rats a closely related analogue of cFP (N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-*p*-aminobenzoate (cFP[Phe])) both potentiated the effects of exogenous bradykinin, and had a hypotensive effect that could be blocked by a bradykinin antagonist (Genden & Molineaux, 1991). The authors of this paper inferred from these findings that cFP[Phe] lowered blood pressure by inhibiting EP 24.15-mediated bradykinin breakdown, and that EP 24.15 is therefore involved in the breakdown of endogenous bradykinin *in vivo*. However, the authors provided no direct evidence that the effects observed were mediated by inhibition of EP 24.15 and not other peptidases, such as angiotensin converting enzyme (ACE), which is known to be the principal mediator of bradykinin degradation (Yang *et al.*, 1970). Indeed, recent studies have shown that this compound can be degraded *in vitro* to form an ACE-inhibiting metabolite (Chappell *et al.*, 1992; Cardozo *et al.*, 1993; Williams *et al.*, 1993).

In the present study we have re-evaluated the hypothesis that cFP inhibits bradykinin breakdown through an interac-

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tion with EP 24.15. Specifically, we have compared the effects of cFP with those of the angiotensin converting enzyme inhibitor captopril, in both conscious rabbits, and in a preparation similar to that employed by Genden & Molineaux (1991), the pentobarbitone anaesthetized rat. In addition, we have tested the interaction of cFP with both crude and purified ACE *in vitro*, using both enzymatic and binding assays. We conclude that cFP inhibits bradykinin breakdown, and lowers blood pressure, by interacting with ACE. These data do not support the notion that EP 24.15 plays a major role in systemic bradykinin metabolism and blood pressure control.

Methods

Twelve male rabbits of a cross-bred English strain and 27 male Sprague-Dawley rats were used. The experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990), and were approved in advance by the Alfred Hospital/Baker Medical Research Institute Animal Experimentation Committee.

Experiments on conscious rabbits

These were designed in a within-subject fashion, so that in each of the three experiments, each rabbit (1.50–2.80 kg (mean 2.25)) was studied on four separate occasions at 7–14 day intervals. The order of these studies was randomised using a 4×4 Latin square design.

Placement of catheters These were inserted on the morning of each study day, and removed at the end of the day. The rabbit was placed in a 15×40×18 cm box fitted with a wire mesh lid and intravascular catheters were inserted under local analgesia (1% lignocaine; Xylocaine, Astra Pharmaceuticals, North Ryde, NSW, Australia). A catheter (Insyte; Deseret Medical, Sandy, Utah, U.S.A.) was inserted into the central ear artery, to measure arterial pressure and to take blood samples when required. A nylon catheter (i.d. 0.50 mm; o.d. 0.63 mm) with a dead space of 40 µl was inserted into the marginal ear vein and advanced 11–15 cm so that the tip was near the right atrium for bolus drug administration. A catheter (Insyte) was also placed in the marginal vein of the other ear for drug infusion. Each rabbit was then allowed 30 min to adapt to its new environment before the experimental procedures began.

Experimental protocols

(1) Effects of cFP (1–25 mg kg⁻¹) on hypotensive responses to exogenous bradykinin and acetylcholine The dose-response relationships were determined for the haemodynamic effects of bradykinin (10, 30, 100, 300 and 1000 ng kg⁻¹) and acetylcholine (30, 300 and 3000 ng kg⁻¹). Both drugs were administered via the right atrial catheter in ascending doses at 4 min intervals, in a volume of 0.1 ml kg⁻¹ of 154 mM NaCl. The two agents were administered alternately except the final two doses of bradykinin, which were in sequence. Five minutes after the last dose of bradykinin, either cFP (1, 5, or 25 mg kg⁻¹) or its vehicle (1.0 ml kg⁻¹ 10% w/v 2-hydroxypropyl-β-cyclodextrin) was administered i.v. After a further 5 min period, dose-response curves to bradykinin and acetylcholine were repeated as above. The effects of a single dose of bradykinin (300 ng kg⁻¹) were also tested 60 and 90 min after cFP or vehicle administration.

(2) Comparisons of the effects of cFP and captopril on haemodynamic responses to angiotensin I and bradykinin On each of the four study days the rabbits received either captopril (0.5 mg plus 10 mg kg⁻¹ h⁻¹, i.v.) or its vehicle (1 ml plus 10 ml kg⁻¹ h⁻¹ 154 mM NaCl, i.v.). Thirty minutes later,

5 mg kg⁻¹ cFP or its vehicle was administered i.v., and after a 5 min equilibration period, dose-response curves to angiotensin I and bradykinin (10, 30 and 100 ng kg⁻¹) were constructed. Angiotensin I and bradykinin were administered via the right atrial catheter in ascending doses, alternating between the two drugs at 4 min intervals.

(3) Comparison of the effects of cFP and captopril on [³H]-bradykinin metabolism In this experiment the effects of cFP and captopril on the breakdown of [³H]-bradykinin were examined. The aim was to use exogenously administered [³H]-bradykinin as a marker for endogenous bradykinin metabolism. As in Experiment 2, on each of the four study days the rabbits received either captopril (0.5 mg plus 10 mg kg⁻¹ h⁻¹) or its vehicle i.v. Thirty minutes later, 5 mg kg⁻¹ cFP or its vehicle was administered i.v. and a control (background) blood sample was taken. One minute after cFP or vehicle administration a bolus of [³H]-bradykinin (1 µCi in 1 ml of 154 mM NaCl) was administered via the right atrial catheter, and 1 ml arterial blood samples were taken 5 and 15 s and 1, 5, 15 and 30 min later. These were collected into chilled syringes containing a cocktail of enzyme inhibitors (see Drugs) over 1–3 s, and analysed by high performance liquid chromatography (h.p.l.c.) as described below.

Rat experiment

The aim was to compare the effects of cFP and captopril on blood pressure and hindlimb vascular resistance, and to test the effect of bradykinin pretreatment on these responses. Rats (*n* = 21; 300–450 g, mean 394 g) were anaesthetized with sodium pentobarbitone (60–120 mg kg⁻¹, i.p.). A tracheal cannula was inserted and catheters were inserted into a femoral artery (for measurement of blood pressure) and a jugular vein (for drug administration). An ultrasonic (transit time) flow probe (type 2S, Transonic Systems, Ithaca, NY, U.S.A.) was placed around the abdominal aorta, below the level of the renal arteries and above the level of the inferior mesenteric artery. A 30 min equilibration period was then allowed before the experimental procedures began.

The effects on blood pressure, heart rate and hindlimb blood flow of bolus injections of cFP (1.5 µmol kg⁻¹ (9.3 mg kg⁻¹), in 1.5 ml kg⁻¹ 10% w/v 2-hydroxypropyl-β-cyclodextrin) and captopril (5 mg kg⁻¹; in 1.0 ml kg⁻¹ 154 mM NaCl) were tested. In some rats these treatments were administered 5 min after pretreatment with bradykinin (50 µg kg⁻¹ in 1 ml kg⁻¹ 154 mM NaCl).

Collection of in vivo data

Recording of haemodynamic variables Arterial pressure was measured continuously throughout the experiments by connecting the arterial catheter to a Statham P23Dc strain gauge, set to zero at the level of the animal's heart. Hindlimb blood flow was measured by connecting the flow probe to an ultrasonic volume flow meter (Model T108; Transonic Systems, Ithaca, NY). The signals were amplified and recorded on a Grass Model 7 polygraph, and sent to an Olivetti M24 computer equipped with an A-D converter which provided mean values for mean arterial pressure (mmHg) and hindlimb blood flow (ml min⁻¹).

Preparation of blood samples for h.p.l.c. Blood samples (1 ml) were collected via an ear artery catheter into chilled syringes, containing a cocktail of enzyme inhibitors (see Drug section), and immediately transferred into chilled 5 ml tubes containing ethylenediaminetetraacetic acid (Sequestrene tube, Johns Disposable Products, South Oakleigh, Australia). Each sample was centrifuged at 3000 r.p.m. for 10 min at 4°C (Minifuge G.L., Heraeus, Germany). The plasma supernatant was aspirated and added to 2 ml methanol (BDH-Chemicals, Australia; analytical grade) to precipitate protein, and cent-

rifuged at 3000 r.p.m. for a further 10 min at 4°C. The supernatant was collected into 5 ml tubes and partially evaporated (to remove the organic component) in a Speedvac concentrator (Savant Instruments, U.S.A.). The remaining aqueous phase (~0.5 ml) was then transferred to Eppendorf tubes. Prior to analytical h.p.l.c., each sample was centrifuged (Eppendorf microcentrifuge Model 5415C, Germany) for 5 min to remove any remaining particulate material. Preliminary studies demonstrated that bradykinin breakdown during the collection and sample preparation process was about 20%. The data presented in Results were not corrected for this systematic error.

H.p.l.c. analysis Plasma extracts were analysed in a Milipore Waters 510 liquid chromatograph system (Milford, MA, U.S.A.), comprising two 510 pumps, a Model 680 gradient controller, a U6K injector and a Model 411 absorbance detector connected to a fraction collector (Pharmacia Fine Chemicals, Australia). The column effluent was monitored at 214 nm. Each 500 µl sample was injected onto a radially compressed C₁₈ Nova Pak reversed-phase column (8 mm × 10 cm) (Waters Associates, U.S.A.). Solvent A was 11 mM trifluoroacetic acid (TFA) and solvent B was 70% acetonitrile containing 11 mM TFA. The separations were achieved using a linear gradient of 3–55% of solvent B over 25 min at a flow rate of 1 ml min⁻¹. Fractions (0.5 ml) were collected into 6 ml glass vials to which 5 ml scintillation fluid (Insta-Gel; Packard, U.S.A.) was added. The vials were then counted for 5 min on a Tri-Carb Model 1900CA liquid scintillation analyser. Synthetic bradykinin (1–9), bradykinin (1–7) and bradykinin (1–5) were used as standards to verify the elution times of the tritiated peptides.

Analysis of in vivo results

The recovery of [³H]-bradykinin, [³H]-bradykinin 1–7 and 1–5, and of non-retained [³H]-bradykinin metabolites over time was quantified graphically. The analysis was complicated by the fact that the time at which blood samples were taken was not always exactly the same. To overcome this problem, we fitted spline curves to the relationships between percentage recovery of ³H (in the form of bradykinin, bradykinin 1–7 and 1–5, and non-retained metabolites) against time in each individual experiment. This was done with the graphical computer software package SIGMAPLOT (Norby *et al.*, 1992). The percentage recoveries at the set time points of 5 and 15 s and at 1, 5, 15 and 30 min, were interpolated (and in a few cases extrapolated) from these curves.

The statistical computer software package SYSTAT (Wilkinson, 1990) was used for statistical analyses. *P* values ≤ 0.05 were considered to be significant. When repeated measures analysis of variance was used *P* values were conservatively adjusted by the Greenhouse-Geisser correction (Ludbrook, 1994). Conventional analysis of variance followed by specific multiple comparisons was also performed in some cases. When more than one *P* value was used from a particular analysis, the Dunn-Sidak correction was applied to protect against the increased risk of type I error (Ludbrook, 1991). All data are expressed as the mean ± s.e.mean.

Enzyme kinetic assay for angiotensin converting enzyme

Rat plasma angiotensin converting enzyme activity was quantified using the synthetic substrate hippuryl-histidyl-leucine (Cushman & Cheung, 1971). Increasing concentrations of cFP or enalaprilat were incubated with 10 µl of plasma and 250 µl of substrate (5 mM in 0.1 M K₂HPO₄ buffer at pH 8.3 containing 300 mM NaCl) for 15 min at 37°C. To a parallel set of assay tubes 10 µM phosphoramidon was also added. Degradation of substrate was terminated by placing tubes in an ice cold bath followed by rapid addition of 1.5 ml of 0.28 M NaOH. Liberated histidyl-leucine was

quantified by fluorometric detection of its product with *o*-phthalaldehyde (Friedland & Silverstein, 1976). Results for plasma samples were read from a standard curve of known concentrations of histidyl-leucine.

Angiotensin converting enzyme radioligand binding studies

Radioiodination of 351A Preparation, purification and stability of radioiodinated 351A have been previously detailed (Jackson *et al.*, 1986). Purified [¹²⁵I]-351A (the *p*-hydroxybenzamidine derivative of lisinopril) was pH adjusted with 10 M NaOH (100 µl per ml of tracer) for subsequent binding experiments at pH 7.0. [¹²⁵I]-351A was stored at –20°C and each batch was used for no longer than 2 months.

Preparation of plasma and solubilized lung membranes Six rats (200–250 g) were killed by decapitation. Trunk blood was collected into chilled tubes containing heparin (500 iu ml⁻¹) and the lungs were removed. Blood was centrifuged at 1,800 g for 15 min at 4°C. Plasma was pooled and lungs were stored at –20°C until required. Membranes were prepared from thawed lungs by a modification of the method of Florentin *et al.* (1984) as described by Perich *et al.* (1992). Lung membranes were solubilized by adding 0.1% v/v Triton X-100, and then mixing on a rotating wheel for 2 h at 4°C. The preparation was centrifuged at 60,000 g at 4°C and the supernatant fraction containing solubilized lung membranes stored at –20°C until assay.

Purification of rat lung ACE The ACE inhibitor, lisinopril, was used to prepare an affinity column and pure ACE was prepared as described by Perich *et al.* (1992). Aliquots were stored at –80°C until required.

Binding studies Binding of [¹²⁵I]-351A to rat plasma and tissue ACE has previously been validated and optimized (Jackson *et al.*, 1986). Plasma and solubilized lung membranes were studied over a range of dilutions to establish the dilution required for approximately 10 to 20% binding of [¹²⁵I]-351A, at which concentration subsequent binding experiments were performed. All assay components were prepared in binding buffer (Jackson *et al.*, 1986). The diluted ACE source (250 µl) was incubated with 50 µl of [¹²⁵I]-351A (10 pg per tube, 40,000 c.p.m. when fresh) and 10 µl of cFP (10 pM to 6 µM final concentration). In a parallel set of assay tubes 10 µM phosphoramidon was also added. All tubes were mixed and equilibrated for 16 h at room temperature. Absolute ethanol (1 ml) was then added and the tubes mixed and then centrifuged at 1,800 g for 5 min at 4°C. The supernatant fraction was discarded and the pellet containing ACE-bound radioligand quantified by gamma-counter (LKB-Wallac Multigamma 1260, Turku, Finland). Displacement of [¹²⁵I]-351A from ACE was analysed by the LIGAND computer programme to derive equilibrium dissociation constants (*K*_D) (Munson & Rodbard, 1980).

Drugs

Drugs used in this study were: phosphoramidon, acetylcholine, angiotensin I (Sigma Chemical Company, U.S.A.); bradykinin (1–9), bradykinin (1–7), bradykinin (1–5) (Auspep, Australia); captopril (Squibb Institute for Medical Research, U.S.A.); 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals Incorporated, U.S.A.); [³H]-bradykinin (25–70 Ci mmol⁻¹; Du Pont Ltd, Australia); 351A, lisinopril, enalaprilat (gifts from Merck Sharpe and Dohme, Rahway, NJ, U.S.A.); hippuryl-histidyl-leucine (Peptide Institute Inc., Osaka, Japan); and N-[1(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (cFP) which was synthesized by the method of Chu & Orłowski (1984).

Blood samples were collected into chilled syringes contain-

ing 110 μ l of a cocktail of enzyme inhibitors comprising: 100 mM ethylenediaminetetra-acetic acid di-sodium salt (Ajax Chemicals, Australia); 10 mM N-ethylmaleimide (Calbiochem, U.S.A.); 1 mg ml⁻¹ bacitracin, 2.31 μ g ml⁻¹ trypsinolol, 10 mM phenylmethylsulphonyl fluoride (Sigma Chemical Company, U.S.A.); 100 μ M lisinopril and 500 μ M amastatin (Auspep, Australia).

Results

Experiments on rabbits

Experiment 1: Effects of cFP (1–25 mg kg⁻¹) on haemodynamic responses to bradykinin and acetylcholine Intravenous administration of bradykinin (10–1000 ng kg⁻¹) was followed by transient falls in arterial pressure (Figures 1–2). Both the magnitude and the duration of these effects increased with dose (P always ≤ 0.003). Intravenous administration of cFP dose-dependently increased both the magnitude and duration of the bradykinin responses (P always ≤ 0.04). A dose of bradykinin which had no effect under control conditions (30 ng kg⁻¹) produced a marked decrease in arterial pressure following cFP treatment (Figures 1–2). Similarly, the hypotensive effects of higher doses of bradykinin (300–1000 ng kg⁻¹) were potentiated and also prolonged after cFP administration (Figures 1–2). A near maximum effect on the response to bradykinin was produced by a dose of 5 mg kg⁻¹ cFP, so this dose was used in subsequent experiments.

The duration of action of cFP was dose-dependent, and was not maximal at a dose of 5 mg kg⁻¹. The hypotensive response to 300 ng kg⁻¹ bradykinin was still potentiated at 60 min, but not 90 min after 5 mg kg⁻¹ cFP. After a dose of 25 mg kg⁻¹ cFP the response to bradykinin was potentiated for at least 90 min (data not shown).

Intravenous administration of acetylcholine was also followed by transient falls in arterial pressure (Figure 1). The response increased both in duration and maximum effect with increasing doses (P always ≤ 0.003). The response to acetylcholine was not influenced by cFP at any of the doses tested (see Figure 1).

Experiment 2: Comparison of the effects of cFP and captopril on blood pressure responses to bradykinin and angiotensin I Depressor responses to bradykinin (30–3000 ng kg⁻¹)

Figure 1 Representative experimental record showing the effects of right atrial administration of (a,b) bradykinin (30 and 300 ng kg⁻¹) and (c) acetylcholine (300 ng kg⁻¹), on arterial pressure before and after right atrial administration of cFP (5 mg kg⁻¹) in a conscious rabbit. $\downarrow$ Denotes time of bradykinin or acetylcholine administration.

were potentiated by cFP (5 mg kg⁻¹) (P always ≤ 0.003) (Figure 3). The effect of captopril was both qualitatively and quantitatively similar to cFP. Responses to bradykinin following combined treatment with captopril and cFP, were indistinguishable from those following treatment with captopril alone (Figure 3).

Intravenous administration of angiotensin I (30–3000 ng kg⁻¹) was followed by dose-dependent rises in arterial pressure ($P = 0.003$). The pressor response at each dose of angiotensin I was reduced after cFP treatment ($P \leq 0.001$). The response to angiotensin I was abolished both following captopril administration, and when cFP and captopril were co-administered (Figure 3).

Experiment 3: Comparison of the effects of cFP and captopril on [³H]-bradykinin metabolism In rabbits receiving vehicle, bradykinin was rapidly broken down. Five seconds after administration 81 $\pm$ 5% of the injected [³H]-bradykinin had already been metabolised. Of this, 60 $\pm$ 9% was in the form of the intermediate metabolites, bradykinin 1–7 and 1–5, while 13 $\pm$ 5% was not retained on the h.p.l.c. column, and thus comprised free proline and/or tritium containing proline metabolites (hereafter referred to as proline). The two peptide fragments (1–7 and 1–5), which eluted together, were then more slowly metabolized to proline. Thus by 30 min

Figure 2 Maximum changes in mean arterial pressure (MAP) following right atrial administration of bradykinin (10–1000 ng kg⁻¹) in conscious rabbits. (○) Control period; (●) after vehicle (a) or cFP 1 mg kg⁻¹ (b), 5 mg kg⁻¹ (c) or 25 mg kg⁻¹ (d) administration. The symbols represent mean $\pm$ s.e. mean values for 4 rabbits.

Figure 3 Maximum changes in mean arterial pressure (MAP) following right atrial administration of bradykinin (10–100 ng kg⁻¹) (a) or angiotensin I (10–100 ng kg⁻¹) (b) in conscious rabbits. Rabbits were pretreated with either: vehicle (○); cFP (5 mg kg⁻¹) (●); captopril (0.5 mg + 10 mg kg⁻¹h⁻¹) (□); or cFP + captopril (■). The symbols represent mean $\pm$ s.e. mean values for 4 rabbits.

after [^3H]-bradykinin administration, proline comprised $54 \pm 15\%$ of the recovered tritium; whilst intact bradykinin and fragments (1-7 and 1-5) comprised only a small proportion of the recovered tritium (3 ± 3 and $14 \pm 8\%$ respectively) (Figure 4). The metabolism of bradykinin was markedly slowed by cFP treatment. At the 5 s time point, native bradykinin recovery was $69 \pm 7\%$ after cFP treatment. The significant effect of cFP on native bradykinin recovery was indicated by both an increase in the amount of bradykinin recovered, averaged over each time point ($P = 0.02$) and an altered slope of the relationship between bradykinin recovery and time (rate of bradykinin breakdown) ($P = 0.003$). In addition, cFP treatment slowed the rate of formation of bradykinin 1-7 and 1-5 ($P = 0.003$). The rate of formation of free proline was not affected by cFP ($P = 0.2$) (Figure 4).

Captopril had almost identical effects to those of cFP. The profile of bradykinin metabolism, following concurrent treatment with cFP and captopril, was not different from that seen when either agent was administered alone. Thus, when compared to captopril alone, cFP plus captopril had no effect on the levels of native bradykinin, bradykinin 1-7 and 1-5, or of free proline averaged across all time points ($P \geq 0.9$). Moreover, the relationships between the recovery of these moieties and elapsed time from [^3H]-bradykinin administration were parallel ($P \geq 0.6$) (Figure 4).

Effects of cFP on resting blood pressure in conscious rabbits The effects of cFP on resting arterial blood pressure were tested in two separate experiments. In Experiment 1, cFP (5 mg kg^{-1}) was administered approximately 10 min after the completion of dose-response curves to bradykinin and acetylcholine. Under these circumstances, there was a small ($-4.1 \pm 2.3 \text{ mmHg}$) fall in arterial pressure which was absent when the vehicle was administered alone ($+0.5 \pm 1.0 \text{ mmHg}$) ($P = 0.05$). In Experiment 2, cFP (5 mg kg^{-1}) or its vehicle was administered to rabbits that had received no previous drug treatment. Under these cir-

cumstances, arterial pressure did not fall following either cFP ($+0.5 \pm 1.0 \text{ mmHg}$) or its vehicle ($+1.5 \pm 1.0 \text{ mmHg}$).

Experiments on rats

Effects of cFP and captopril on blood pressure and hindlimb vascular resistance in anaesthetized rats Intravenous administration of cFP (9.3 mg kg^{-1} , $n = 5$) was followed by a sustained fall in mean arterial pressure. The maximum fall averaged $12.6 \pm 3.3 \text{ mmHg}$ (Figure 5). Captopril (5 mg kg^{-1} , $n = 5$) administration was followed by a maximum fall of mean arterial pressure of $23.0 \pm 6.2 \text{ mmHg}$ (Figure 5). The depressor effects of cFP and captopril were accompanied by decreases in hindlimb vascular resistance of 25 ± 6 and $23 \pm 9\%$ of their baseline levels respectively (data not shown).

Intravenous administration of bradykinin ($50 \mu\text{g kg}^{-1}$, $n = 11$) was followed by transient falls in arterial pressure ($39.0 \pm 4.7 \text{ mmHg}$) and hindlimb vascular resistance ($51 \pm 4\%$ of its baseline level). Four minutes later, arterial pressure had returned to its pretreatment level, and hindlimb vascular resistance was $22 \pm 4\%$ higher than its pretreatment level. When cFP ($n = 5$) or captopril ($n = 6$) was administered 5 min after bradykinin, arterial pressure fell by 30.2 ± 4.4 and $37.8 \pm 8.8 \text{ mmHg}$ respectively, and hindlimb vascular resistance fell by 41 ± 6 and $44 \pm 8\%$ respectively, significantly more than was the case when they were administered to naive rats ($P \leq 0.005$). When cFP was administered after both bradykinin and captopril ($n = 5$), no depressor effect was observed (data not shown).

Inhibition of ACE by cFP in vitro CFP displaced [^{125}I]-351A from both solubilized rat lung membrane ACE and rat plasma ACE ($pK_D = 6.85 \pm 0.05$, 5.93 ± 0.16), although its apparent affinity was much lower than that of the ACE inhibitor enalaprilat ($pK_D = 9.97 \pm 0.09$ in plasma). Co-incubation with the EP 24.11 inhibitor phosphoramidon ($10 \mu\text{M}$) reduced the apparent affinity of cFP for [^{125}I]-351A-labelled sites in rat lung ($pK_D = 5.28 \pm 0.41$) (Figure 6) and to a lesser extent in rat plasma ($pK_D = 5.18 \pm 0.43$). When purified rat lung ACE was tested, only 30% displacement

Figure 4 Effects of cFP and captopril on percentage of recovered ^3H in the form of native bradykinin (a), bradykinin 1-7 and 1-5 (b), and free proline (c) in conscious rabbits. The treatments were: vehicle (○), cFP (5 mg kg^{-1}) (●), captopril ($0.5 \text{ mg} + 10 \text{ mg kg}^{-1} \text{ h}^{-1}$) (□), and cFP + captopril (■). The left and right hand panels show responses in rabbits receiving vehicle and captopril respectively. The symbols represent mean $\pm$ s.e. mean for 4 rabbits.

Figure 5 Levels of arterial pressure in anaesthetized rats before and after intravenous administration of cFP (9.3 mg kg^{-1}) or captopril (5 mg kg^{-1}). The left panels show the effects of cFP (a) and captopril (b) in experimentally naive rats. The right panels show the effects of cFP (c) and captopril (d) in rats treated 5 min previously with bradykinin ($50 \mu\text{g kg}^{-1}$). Symbols represent the mean $\pm$ s.e. mean levels for 5-6 rats, of arterial pressure averaged over 60 s periods.

Figure 6 Displacement of [125 I]-351A from rat lung ACE by cFP. Solubilized rat lung membranes were incubated with 10 pg [125 I]-351A and cFP (10 fM–6 μ M) in the absence (O) or presence (●) of 10 μ M phosphoramidon. Phosphoramidon reduced the potency of cFP in displacing [125 I]-351A by nearly 40 fold.

was observed at the highest concentration of cFP used (2.5 μ M). The enzymatic activity of plasma ACE was inhibited by cFP ($pK_i = 5.61 \pm 0.13$), although its potency was again much lower than that of enalaprilat ($pK_i = 8.34 \pm 0.09$). Addition of phosphoramidon (10 μ M) to the incubate reduced the potency of cFP at least 4 fold ($pK_i > 5$).

Discussion

The results of the present study confirm and extend those of Yang *et al.* (1994), who recently found that in anaesthetized rats, the phenylalanine substituted analogue of cFP (N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-*p*-aminobenzoate (cFP[Phe])) had similar effects to the ACE inhibitor, enalaprilat. They found that cFP[Phe] potentiated the hypotensive action of bradykinin and inhibited the pressor effect of angiotensin I, but had no further action when administered after enalaprilat. We have shown not only that the specific EP 24.15 inhibitor cFP potentiates the depressor effect of bradykinin, but have also provided direct evidence that it inhibits bradykinin breakdown in the conscious rabbit. It also appears to inhibit conversion of angiotensin I to its bioactive metabolite angiotensin II, since it markedly attenuates the haemodynamic response to angiotensin I. In both respects, its effects are quantitatively and qualitatively similar to those of the angiotensin converting enzyme inhibitor, captopril, and the effects of cFP and captopril are not additive. Given that captopril has no inhibitory effects on EP 24.15 (Acker *et al.*, 1987), it seems reasonable to conclude that the actions of cFP *in vivo* are not mediated by EP 24.15 inhibition, but rather by inhibition of ACE. Furthermore, we have shown in a series of *in vitro* experiments, that cFP does bind to and inhibit ACE. The apparent increased affinity of cFP for ACE in the absence of phosphoramidon suggests that this interaction is at least partly dependent on the conversion of cFP by a phosphoramidon-sensitive metalloendopeptidase to an ACE inhibiting product. We also conclude that the blood-pressure lowering effects of cFP in conscious rabbits and anaesthetized rats are entirely attributable to ACE inhibition, since they are similar to those of captopril and at least in the rat, cFP has no further blood pressure lowering effect when it is administered after captopril.

Following intravascular administration, exogenous bradykinin is rapidly degraded. Pesquero *et al.* (1992) found in conscious rats that exogenous bradykinin was at least 50 fold more potent when injected intra-arterially than when injected intravenously. They inferred from this that 98% of

bradykinin inactivation occurred in the pulmonary circulation. This breakdown process involved rapid conversion of intact bradykinin to bradykinin 1-7 and 1-5. The present study confirms and extends these observations, using a dose of [3 H]-bradykinin (~ 8 ng kg^{-1}) that would not alter arterial pressure in intact rabbits, and which can therefore be used as a marker for the metabolic fate of endogenous bradykinin. Basal plasma levels of bradykinin have not been determined in rabbits, but in rats they have been found to range from 2–35 pg ml^{-1} (Salgado *et al.*, 1986; Nakagawa & Nasjliti, 1989; Campbell *et al.*, 1993). Based on an estimated blood volume in the rabbit of 65 ml kg^{-1} (Schalm, 1965), the peak plasma level of [3 H]-bradykinin in the present experiment would have been in the order of 100 pg ml^{-1} .

In vehicle-treated rabbits, $81 \pm 5\%$ of right atrially administered [3 H]-bradykinin was degraded 5 s after its injection. Most of this was in the form of bradykinin 1-7 or 1-5 (the chromatographic separation procedure only poorly resolves these two peptides). CFP administration dramatically increased bradykinin recovery (to $69 \pm 7\%$ at 5 s) and significantly slowed the formation of bradykinin metabolites (1-7 and 1-5).

In conscious rabbits, administration of cFP (1–25 mg kg^{-1}) dose-dependently potentiated the hypotensive response to bradykinin. The effects of cFP were specific for bradykinin and not attributable to a general increase in sensitivity to vasodilator agents, since cFP had no effect on the hypotensive response to acetylcholine. However, we also found that cFP markedly attenuated the pressor response to angiotensin I, indicating that it inhibited the conversion of angiotensin I to its active metabolite angiotensin II.

The effects of cFP on the haemodynamic responses to bradykinin and angiotensin I, and on the metabolism of a bolus dose of [3 H]-bradykinin, were qualitatively and quantitatively similar to those of the angiotensin converting enzyme inhibitor captopril. Moreover, the effects of combined treatment with both agents were not greater than treatment with either agent alone. Given that captopril has negligible activity as an inhibitor of EP 24.15 (Acker *et al.*, 1987), if cFP were having an action on bradykinin metabolism in addition to inhibition of ACE, the effects of combined treatment with cFP and captopril should be greater than those of either agent alone. Thus, these data provide strong evidence that cFP and captopril act at the same level to inhibit both bradykinin breakdown and conversion of angiotensin I to angiotensin II in the rabbit; that is, inhibition of angiotensin converting enzyme.

This conclusion seems at odds with the observation that cFP has negligible ACE-inhibiting activity when tested with purified or recombinant ACE preparations (Prof. Pierre Corvol, personal communication). However, two recent reports have provided evidence that cFP[Phe], which also has negligible ACE-inhibiting activity when tested with purified ACE preparations (Williams *et al.*, 1993), can be converted to a potent inhibitor of angiotensin converting enzyme through the actions of endopeptidase 24.11 (Cardozo & Orlowski, 1993; Williams *et al.*, 1993). The ACE inhibiting product of cFP[Phe], N-[1(R,S)-carboxy-3-phenylpropyl]-Ala-Ala, is structurally similar to enalaprilat, inhibiting ACE with a K_i of about 40 nM (Cardozo & Orlowski, 1993; Williams *et al.*, 1993). Our experiments using crude and purified ACE preparations suggest that cFP itself is similarly susceptible to degradation *in vitro* to form N-[1(R,S)-carboxy-3-phenylpropyl]-Ala-Ala. We found that cFP displaced [125 I]-351A from ACE binding sites in preparations of rat lung membrane and rat plasma with apparent micromolar affinity but had negligible affinity for purified rat lung ACE. CFP also inhibited rat plasma ACE activity at micromolar concentrations. The affinity and activity of cFP at these sites was dramatically reduced by the addition of phosphoramidon. Phosphoramidon potentially inhibits the activity of a wide range of metalloendopeptidases including endopeptidase 24.11 (Williams *et al.*, 1993), endothelin converting enzyme

(Ohnaka *et al.*, 1993) and endothelin 1 inactivating enzyme (Janas *et al.*, 1994). Therefore, the precise nature of the product(s) of cFP metabolism *in vivo*, and the enzyme(s) involved, remain to be determined.

Our conclusion that cFP acts as an ACE inhibitor *in vivo* also seems at odds with the observation by Genden & Molineaux (1991) of a transient yet profound fall in arterial pressure following intravenous administration of cFP[Phe] to anaesthetized rats. In the present study we found that administration of cFP to conscious, experimentally naive rabbits did not lower their arterial pressure. We did find, however, that cFP had a small yet statistically significant depressor effect in rabbits that had been administered bradykinin and acetylcholine. These observations in rabbits led us to compare the effects of cFP and captopril in anaesthetized rats, under conditions similar to those used in the study by Genden & Molineaux (1991). We observed modest yet sustained falls in arterial pressure after administration of either cFP (13 mmHg) or captopril (23 mmHg), consistent with the results of other studies in which ACE inhibitors have been shown to reduce moderately arterial pressure in anaesthetized normotensive rats (Mattson & Roman, 1991). We also found that the hypotensive effects of both cFP and captopril were markedly potentiated by pretreatment with a single dose of bradykinin. Indeed, the magnitudes (30–40 mmHg) of these depressor responses were similar to the magnitude of the depressor effect observed by Genden & Molineaux (1991). Ours is not a particularly novel finding, since bradykinin pretreatment has been previously shown to potentiate the hypotensive effects of ACE inhibition in conscious rats (Van Den Buuse & Kerkhoff, 1991). It does,

however, reinforce the point that the response to ACE inhibition is dependent upon the prevailing haemodynamic circumstances, not the least of which is probably the level of plasma renin activity. Our observation that cFP produced no further fall in arterial pressure following captopril administration provides strong evidence that the hypotensive effect of cFP is mediated through inhibition of ACE.

In conclusion, these results show that the effects of cFP on systemic bradykinin metabolism and arterial pressure can be attributed to inhibition of angiotensin converting enzyme rather than EP 24.15. The possibility that EP 24.15 plays some role in systemic bradykinin metabolism and blood pressure control can therefore neither be confirmed nor denied until more stable inhibitors become available that do not have metabolites with angiotensin converting enzyme inhibiting activity. A recent report, however, demonstrated a natriuretic effect of cFP[Phe] which though blunted, was not abolished by pretreatment with the ACE inhibitor enalaprilat (Yang *et al.*, 1994). Whether or not this observation reflects a specific role of EP 24.15 in the kidney also awaits the development of more stable inhibitors of EP 24.15.

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