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Role of bradykinin receptors in the renal effects of inhibition of angiotensin converting enzyme and endopeptidases 24.11 and 24.15 in conscious rabbits

[1]Fumihiro Tomoda, Rebecca A. Lew, A. Ian Smith, Anna C. Madden & [2]Roger G. Evans

Baker Medical Research Institute, Prahran, Victoria, Australia.

1 We tested the effects on systemic haemodynamics and renal function, of inhibition of endopeptidase (EP) 24.15 (E.C. 3.4.24.15), in conscious uninephrectomized rabbits in which the activities of angiotensin converting enzyme (ACE, E.C. 3.4.15.1) and neutral endopeptidase (EP 24.11, E.C. 3.4.24.11) were already inhibited. To test the role of bradykinin B_2-receptors in mediating the effects following inhibition of these enzymes, the antagonist Hoe 140 was used.

2 Hoe 140 (0.1 mg kg^{-1}, i.v.) did not affect resting mean arterial pressure or heart rate, but antagonized the depressor effect of right atrial administration of bradykinin. The dose-response curve for bradykinin was shifted more than 1000 fold to the right for more than 4 h. Hoe 140 approximately doubled resting urine flow and increased fractional Na^+ excretion from 4.2 to 6.0%; consistent with the hypothesis that it exerts a partial agonist effect on the kidney.

3 Combined inhibition of ACE (captopril; 0.25 mg kg^{-1} plus 0.2 mg $kg^{-1}h^{-1}$) and EP 24.11 (SCH 39370; 3 mg kg^{-1} plus 3 mg $kg^{-1}h^{-1}$) was followed by a sustained reduction in arterial pressure (-6 ± 2 mmHg) and increase in heart rate (35 ± 7 beats min^{-1}). There was a small increase in renal blood flow (by 6.5 ± 3.2% relative to vehicle-treatment) without a change in glomerular filtration rate, and about a 150% increase in Na^+ excretion. Hoe 140 (0.1 mg kg^{-1}, i.v.) pretreatment did not influence the renal effects of captopril and SCH 39370, although it did appear to blunt their hypotensive and tachycardic effects.

4 When EP 24.15 was inhibited with N-[1-(**R,S**)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (cFP-AAY-pAB; 5 mg kg^{-1} plus 3 mg $kg^{-1}h^{-1}$, i.v.) in rabbits pretreated with captopril and SCH 39370, no changes in systemic haemodynamics or renal function were observed.

5 We concluded that in conscious uninephrectomized rabbits, EP 24.15 does not play a major role in modulating renal function, at least under conditions where ACE and EP 24.11 are already inhibited. In contrast, ACE and/or EP 24.11 do modulate renal function in this model, but their influences are mediated chiefly through metabolism of peptides other than bradykinin.

Keywords: Angiotensin converting enzyme; bradykinin; Hoe 140; metalloendopeptidase 24.11; metalloendopeptidase 24.15; conscious rabbit; renal function

Introduction

Metalloendopeptidase 24.15 (E.C.3.4.24.15), a zinc-dependent neutral endopeptidase (EP), was first isolated from soluble fractions of brain homogenates by Orlowski *et al.* (1983). It is widely distributed in mammalian tissue, being most abundant in the testes, brain and pituitary, but is also present in the liver, kidney, lung and spleen (Orlowski *et al.*, 1988). *In vitro*, it cleaves a number of substrates, including bradykinin, some opioid peptides, neurotensin, and gonadotrophin releasing hormone (Chu & Orlowski, 1985). Less is known of the physiological functions of this enzyme, though recent experiments have provided evidence for roles in degradation of gonadotrophin releasing hormone within the pituitary (Lasdun *et al.*, 1989; Lasdun & Orlowski, 1990; Lew *et al.*, 1994) and along with EP 24.16 (E.C.3.4.24.16), in degradation of neurotensin in peripheral tissues (Barrelli *et al.*, 1994; Vincent *et al.*, 1995).

One of the major impediments to the advancement of our understanding of the functions of EP 24.15 has been the lack of potent, selective and stable inhibitors of this enzyme. We have focussed our interests on a substrate-related inhibitor of EP 24.15, N-[1-(**R,S**)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (cFP-AAY-pAB) (Chu & Orlowski, 1984; Orlowski *et al.*, 1988). Although this compound, and its phenylalanine substituted analogue cFP-AAF-pAB are potent (K_i values of 16 and 27 nM respectively) and selective inhibitors of EP 24.15, they are unfortunately unstable *in vivo*, being broken down rapidly to produce N-[1-(**R,S**)-carboxy-3-phenylpropyl]-Ala-Ala (cFP-AA), a potent inhibitor of angiotensin converting enzyme (ACE; E.C.3.4.15.1) (Lasdun *et al.*, 1989; Chappell *et al.*, 1992; Cardozo & Orlowski, 1993; Williams *et al.*, 1993; Telford *et al.*, 1995). The utility of these inhibitors as pharmacological tools for studying the physiology of EP 24.15 *in vivo* is thus limited by their short half life and the fact that experiments performed without concomitant inhibition of ACE are difficult to interpret.

Recently much interest has been focussed on the renal kallikrein-kinin system as a target for the development of novel antihypertensive agents (see, Majima & Katori, 1995). Both ACE and EP 24.11 (E.C.3.4.24.11) appear to play important roles in the metabolism of bradykinin within the kidney (Seymour *et al.*, 1994). Since EP 24.15 is found in the kidney (Chu & Orlowski, 1985), and since bradykinin may be an important substrate for this enzyme (Orlowski *et al.*, 1989), the potential role of EP 24.15 in renal bradykinin metabolism also merits investigation. Yang *et al.* (1994) tested the effect of cFP-AAF-pAB on renal function in anaesthetized rats pretreated with the ACE inhibitor enalaprilat. They found that under these experimental conditions cFP-AAF-pAB increased glomerular filtration rate, urine output, and the urinary excretion of Na^+ and K^+. This effect was attributed to inhibition of EP 24.15, or some other non-ACE peptidase. These studies did not

[1]Present address: Second Department of Internal Medicine, Toyama Medical & Pharmaceutical University, 2630 Sugitani, Toyama, Japan.
[2]Author for correspondence at present address: Department of Physiology, Monash University, Clayton, Victoria, 3168, Australia.

exclude the possibility that the ACE-independent renal effects of cFP-AAF-pAB were mediated by inhibition of EP 24.11 rather than EP 24.15. Indeed, it is possible that the dose administered ($\sim$10 μmol kg^{-1}, i.v.) might have inhibited EP 24.11, since we calculate that it would have resulted in peak plasma levels in the order of 200 μM (the K_i of cFP-AAF-pAB for EP 24.11 is 17 μM; Orlowski *et al.*, 1988). This possibility is supported by the results of our own experiments in conscious rabbits, in which we found that a similar i.v. dose of cFP-AAY-pAB (8.3 μmol kg^{-1}/5 mg kg^{-1}), although rapidly cleared from the circulation, resulted in peak plasma levels of 137$\pm$31 μM (Lew *et al.*, 1996). Therefore, in the present study we have tested the renal effects of cFP-AAY-pAB in rabbits pretreated with both the ACE-inhibitor, captopril and the EP 24.11 inhibitor, SCH 39370 (Sybertz *et al.*, 1989), both of which have negligible activity as inhibitors of EP 24.15 (Acker *et al.*, 1987; R.A. Lew, unpublished observation). To determine the role of bradykinin in the effects of inhibition of ACE, EP 24.11 and EP 24.15, we also performed this experiment in rabbits pretreated with the bradykinin B_2 receptor antagonist, Hoe 140 (Wirth *et al.*, 1991; Rhaleb *et al.*, 1992).

Methods

Eleven rabbits of a cross-bred English strain were used, weighing 2.0–2.6 kg (mean 2.3) at the time of their first study. 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 Ethics Committee. All experiments were designed in a within-subject fashion, so that within each protocol each rabbit was studied on 3–5 separate occasions at 7–14 day intervals. The order of these studies was randomized.

Experimental protocols

(1) Effects of Hoe 140 on depressor responses to bradykinin

The aim of this experiment was to determine a suitable dose of Hoe 140 for use in subsequent experiments. Although there has been a previous report of the use of this compound in rabbits (Hartman *et al.*, 1993), the potency and duration of action of Hoe 140 as a bradykinin B_2-antagonist has not been formally tested in this species. The effects of this compound on resting arterial pressure and heart rate were also tested in order to determine whether the partial agonist effect of this compound, reported by others in conscious dogs (Wirth *et al.*, 1991) could be seen under our experimental circumstances. Each of four rabbits was studied on 4 separate occasions. Following completion of the minor preparative procedures (see below), each rabbit was allowed at least 30 min to adapt to its new environment before the experimental procedures began. The effects of right atrial administration of ascending bolus doses of bradykinin (1 ng kg^{-1} to 100 μg kg^{-1}, or until mean arterial pressure fell by 35 mmHg or more) were tested. Each rabbit was then treated, on separate days, with either Hoe 140 (0.01, 0.1 or 1.0 mg kg^{-1}, i.v.) or its vehicle (5% w/v dextrose, 0.2 ml kg^{-1}). Resting arterial pressure and heart rate were monitored before and after administration of Hoe 140 or its vehicle, and the responses to bradykinin were retested 1 h, 2 h, 3 h and 4 h after administration of Hoe 140 or its vehicle.

(2) Renal effects of inhibition of EP 24.15

General The aims of this experiment were to determine the systemic haemodynamic and renal effects of (i) the B_2 antagonist Hoe 140, (ii) combined inhibition of ACE and EP 24.11, and (iii) inhibition of EP 24.15 during established inhibition of ACE and EP 24.11. A further aim was to (iv) determine the roles of bradykinin B_2 receptors in mediating the effects of inhibition of these 3 enzymes. Each of 7 rabbits was studied up to 5 times (2 rabbits did not complete all 5 studies). One study served as a time control, while in the other 4 studies, all rabbits were treated with inhibitors of ACE and EP 24.11 and were either treated with the EP 24.15 inhibitor cFP-AAY-pAB or its vehicle, and the bradykinin antagonist Hoe 140 or its vehicle.

Immediately following insertion of the bladder catheter (see below), the rabbits were given a mild volume and salt load via an i.v. infusion of 0.9% w/v NaCl at a rate of 0.741 ml min^{-1} kg^{-1} for 30 min. Blank urine and blood (1 ml) samples were collected during this period. Bolus doses of [^{3}H]-inulin (4 μCi) (DuPont NEN, Melbourne, Australia), para-aminohippuric acid (PAH, 20 mg) (Sigma Chemical Co., St Louis, U.S.A.) and LiCl (25 mg) (Merck, Germany) were then administered i.v. in 2 ml of 5% w/v dextrose. For the remainder of the experiment the rabbits received an i.v. infusion of 0.18% w/v NaCl in 4% w/v dextrose, infused at a rate of 0.18 ml min^{-1} kg^{-1}. This solution also contained 0.3 μCi ml^{-1} [^{3}H]-inulin (dose = 54 nCi min^{-1} kg^{-1}), 2 mg ml^{-1} PAH (dose = 0.36 mg min^{-1} kg^{-1}) and 0.25 mg ml^{-1} LiCl (dose = 45 μg min^{-1} kg^{-1}). Using this protocol the plasma levels of [^{3}H]-inulin, PAH and Li^+ remained stable for the 80 min of the study, at about 25000 d.p.m. ml^{-1}, 13 μg ml^{-1}, and 0.45 mM respectively. Ninety minutes after the beginning of this infusion, the bladder was emptied by injecting 5 ml of air into the bladder catheter, followed by 10 ml of deionised water to flush out any remaining urine. The first of four clearance periods, each of 20 min duration, then commenced. Blood samples (1 ml), for determination of plasma [^{3}H]-inulin, PAH, Na^+, K^+ and Li^+ were taken just before the start of the first and third clearance period, and at the completion of the fourth clearance period. Urine (for the same measurements) was collected over each of the 20 min clearance periods; the bladder being emptied as above at the end of each period. Urine volume was measured gravimetrically. Blood samples (2 ml) were also collected for determination of cFP-AAY-pAB and SCH 39370 levels, 10 min after starting the second clearance period, and at the end of the third and fourth clearance periods. Blood volume was replaced whenever blood samples were taken by intravenous administration of an equivalent volume of polygeline/electrolyte solution (Haemaccel, Hoechst Australia Pty Ltd, Victoria).

Drug treatments Each rabbit was administered either Hoe 140 (0.1 mg kg^{-1}) or its vehicle (1 ml kg^{-1}, 5% dextrose) 90 min before the start of the first clearance period. At the end of the first clearance period, treatment with either captopril (0.25 mg kg^{-1} plus 0.2 mg kg^{-1} h^{-1}) and SCH 39370 (3 mg kg^{-1} plus 3 mg kg^{-1} h^{-1}) or their vehicle (0.115 M phosphate buffer pH 7.4; 1 ml kg^{-1} plus 5 ml kg^{-1} h^{-1}) commenced. At the end of the second clearance period, each rabbit was treated with either cFP-AAY-pAB (5 mg kg^{-1} plus 3 mg kg^{-1} h^{-1}) or its vehicle (1 ml kg^{-1} plus 0.6 ml kg^{-1} h^{-1}, 10% 2-hydroxypropyl-β-cyclodextrin).

Surgical procedures

Implantation of flow probes In preparation for the renal function experiment (Protocol 2) each rabbit was equipped with a transit time flow probe (type 2SB, Transonic Systems, Ithaca, U.S.A.) around the left renal artery. Anaesthesia was induced with i.v. propofol (10 mg kg^{-1}, Diprivan, ICI, Victoria, Australia), and after endotracheal intubation maintained with halothane (1–4%; Fluothane, ICI, Victoria, Australia). Via a midline incision the left renal artery was exposed for implantation of the flow probe, which was secured in place around the renal artery with a silicone sheet (0.25 mm thickness, Future Medics, Victoria, Australia). The cable of the flow probe was tunnelled under the skin so that the plug lay subcutaneously between the rabbit's shoulder blades. The right kidney was removed. Post-operative medications comprised i.m. trimethoprim (16 mg) and sulphadiazine (80 mg) (Tribrissen; Jurox, NSW, Australia) and s.c. flunixin (5 mg; Finadyne, Heriot, Agvet, Victoria, Australia).

Minor procedures on study days 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. For the experiment in which the potency and duration of action of the bradykinin antagonist Hoe 140 were studied (Protocol 1), 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. For the renal function study (Protocol 2), a catheter (Insyte) was placed in the marginal ear vein for drug infusion and the plug of the flow probe was retrieved from its subcutaneous position under local analgesia (1% lignocaine). Then, under brief general anaesthesia (5 – 10 mg kg^{-1} propofol, i.v.) a bladder catheter was inserted (8 F-gauge latex, infant urological Foley catheter; Rusch, High Wycombe, Buckinghamshire, U.K.). At the completion of the study day the catheters were removed and the plug of the flow probe was secured in its subcutaneous position under local analgesia (1% lignocaine).

Data collection

Recording of haemodynamic variables Arterial pressure was measured by connecting the arterial catheter to a Statham P23Dc strain gauge, set to zero at the level of the animals heart. Heart rate was measured by a rate meter (Baker Medical Research Institute model 183; Prahran, Victoria, Australia) activated by the pressure pulse. Renal blood flow was measured by connecting the flow probe to an ultrasonic volume flow meter (Model T108; Transonic Systems, Ithaca, NY, U.S.A.). The signals were amplified and recorded on a Grass Model 7 polygraph (Quincy, MA, U.S.A.), and sent to an Olivetti M24 computer equipped with an A – D converter which provided 2 s or 20 s mean values for arterial pressure (mmHg), heart rate (beats min^{-1}) and renal blood flow (ml min^{-1}).

Analysis of urine and blood samples for renal function measurements

Clearance measurements These have been described in detail previously (Evans *et al.*, 1994; Evans & Anderson, 1995). Briefly, Na^+, K^+ and Li^+ concentrations in plasma and urine were measured by flame photometry (Instrument Laboratory 943, Italy), PAH concentrations were measured by the method of Smith *et al.* (1945), and [^{3}H]-inulin levels were evaluated by liquid scintillation counting.

Clearance calculations [^{3}H]-inulin clearance was used to estimate glomerular filtration rate (see Evans *et al.*, 1994). Fractional solute excretion was calculated as the ratio of solute clearance to [^{3}H]-inulin clearance. Fractional Na^+ reabsorption in the proximal and distal tubules were estimated as 100 × (1-Li^+ clearance/inulin clearance) and 100 × (1-Na^+ clearance/Li^+ clearance), respectively. Although Li^+ clearance does not provide a direct measure of proximal tubular Na^+ reabsorption, it remains the most convenient marker of proximal tubular Na^+ handling, particularly in conscious animals (see Koomans *et al.*, 1989). Renal blood flow was estimated by two independent methods; ultrasonic flowmetry and PAH clearance (corrected for haematocrit). As we found previously in a study using conscious dogs (Evans & Anderson, 1995), there were systematic differences between the levels of renal blood flow estimated by the two methods, which we have attributed to variability in the calibration of these probes for chronic *in vivo* use (see Evans & Anderson, 1995). Therefore, to provide both accurate and precise estimates of renal blood flow, we corrected all doppler flowprobe measurements by normalising them to the average levels of renal blood flow estimated by the PAH clearance technique on the particular study day.

Determination of blood levels of SCH 39370 and cFP-AAY-pAB

Blood sample processing Arterial blood samples (2 ml) were collected into chilled syringes, and immediately transferred into chilled 5 ml heparinized tubes. Each sample was centrifuged at 3000 r.p.m. for 10 min at 4°C (Minifuge G.L, Heraeus, Germany). One ml of the plasma supernatant was aspirate and added to 5 ml methanol (BDH-Chemicals, Australia; analytical grade) to precipitate protein, and centrifuged 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. Standard curves for SCH 39370 and cFP-AAY-pAB were constructed by adding known amounts of each compound to 2 ml blood samples freshly taken from experimentally naive rabbits, to achieve final concentrations of 0, 0.1, 0.3, 1, 3 and 30 μM SCH 39370 and 0, 0.01, 0.1, 1.0, 10 and 100 μM cFP-AAY-pAB; these standards were processed and assayed in parallel with the experimental samples.

Assay of blood levels of SCH 39370 Blood levels of SCH 39370 were determined by assessing the ability of these plasma extracts to inhibit the degradation of the EP 24.11 substrate cFP-AAY-pAB by kidney membranes, a rich source of EP 24.11. Rabbit kidney membranes were prepared by homogenization in 0.2 M N-tris[hydroxymethyl]-methyl-2-aminoethanesulphonic acid (TES) buffer, pH 7.4, at 4°C, followed by ultracentrifugation (100 000 *g*, 60 min, 4°C). The resultant pellet was resuspended in fresh TES buffer, and recentrifuged. The second pellet was again resuspended in TES buffer at a concentration of 30 mg ml^{-1}, and frozen in aliquots at −70°C until needed. The plasma extracts were reconstituted to 1 ml, and aliquots (100 μl) were added to 6 μg kidney membranes and 5 μg cFP-AAY-pAB in a final volume of 150 μl TES buffer, and incubated at 37°C for 60 min. Following incubation, the enzymatic reactions were stopped by addition of 600 μl methanol/1% trifluoroacetic acid (TFA). Samples were centrifuged in a table top microfuge (14,000 r.p.m., 5 min) to precipitate protein, and the supernatants dried by vacuum centrifugation (Speed-Vac, Savant) and reconstituted in 250 μl h.p.l.c. solvent A (0.08% TFA) before analysis by h.p.l.c. (see below).

High performance liquid chromatography (h.p.l.c.) for quantification of cFP-AAY-pAB levels

Samples were injected on to a Novapak C18 column (8.00 mm i.d. × 100 mm) contained within a radial compression module (Waters Associates), and constituents were eluted from the column by a linear (30 min) gradient from 3% – 70% solvent B (70% CH_3CN/0.08% TFA) at a flow rate of 1 ml min^{-1}. Chromatography was performed with a Waters h.p.l.c system, consisting of an automated gradient controller (Model 680), two Model 510 pumps, a Model 712 (WISP) autosampler, and a Model 441 detector (214 or 254 nm), and the data analysed by Waters Maxima computer software.

The extent of cFP-AAY-pAB degradation by kidney membranes, and in turn, its protection by inhibitors of EP 24.11, was assessed by summation of the areas of the two absorbance peaks representing the intact cFP-AAY-pAB stereoisomers. Standard curves relating the total cFP-AAY-pAB peak area to the EP 24.11 inhibitor concentration were constructed for SCH 39370, and the inhibitor concentrations present in the experimental samples were determined from these curves. The level of cFP-AAY-pAB itself in blood samples was assessed in an identical manner, with peak areas related to concentration using the standards, followed by estimation of levels in the experimental samples.

Statistical analysis

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 was also performed when appropriate. 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 $\pm$ 1 s.e.mean.

Drugs

Drugs used in this study were: N-[N-[1-(S)-carboxyl-3-phenylpropyl]-(S)-phenylalanyl]-(S)-isoserine (SCH 39370) (Sybertz *et al.*, 1989) (a gift from Schering-Plough Pty Ltd, Baulkham Hills, Australia); D-Arg-(Hyp^3,Thi^5,D-Tic^7,Oic^8 bradykinin) (Hoe 140) (Wirth *et al.*, 1991; Rhaleb *et al.*, 1992) (a gift from Hoechst Australia Ltd, Melbourne, Australia); bradykinin (Auspep, Australia); captopril (Squibb Institute for Medical Research, U.S.A.); 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals Incorporated, U.S.A.); and N-[1-(**R,S**)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (cFP-AAY-pAB) (Orlowski *et al.*, 1988) which was synthesized by the method of Chu & Orlowski (1984).

Results

(1) Effects of Hoe 140 on resting blood pressure and heart rate, and on the haemodynamic response to bradykinin

Bolus doses of Hoe 140 (0.01 – 1.0 mg kg^{-1}) or its vehicle had no discernible effect on resting levels of mean arterial pressure or heart rate. Thus, levels of these variables in the 1 min period before administration of Hoe 140 were not different from the levels in the 1 min following bolus administration (P always > 0.24) (data not shown).

Right atrial administration of bradykinin (1 – 1000 ng kg^{-1}) produced transient and dose-dependent reductions in arterial pressure. The response to bradykinin was not affected by administration of the vehicle in which Hoe 140 was dissolved. In contrast, Hoe 140 caused profound and prolonged inhibition of the responses to bradykinin. Following all three doses of Hoe 140 (0.01, 0.1 and 1.0 mg kg^{-1}), the response to 1000 ng kg^{-1} bradykinin was completely abolished for up to 4 h, and the dose-response curve for bradykinin was shifted to the right at least 1000 fold (Figure 1). There did, however, appear to be some recovery of the response to the highest doses of bradykinin (10 and 100 μg kg^{-1}) 3 – 4 h after the lowest dose (0.01 mg kg^{-1}) of Hoe 140, but not after the higher doses. Therefore, we chose to use the intermediate dose of 0.1 mg kg^{-1} in our subsequent experiment.

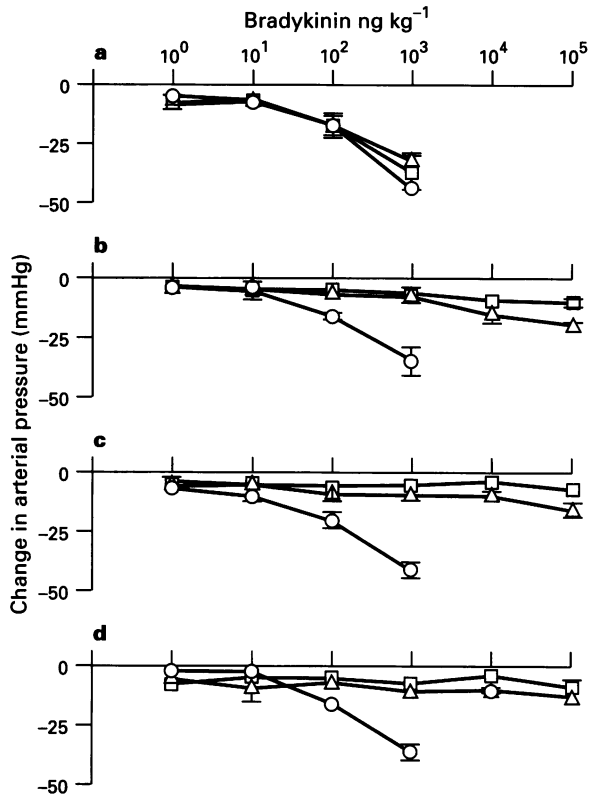

Figure 1 Changes in mean arterial pressure following right atrial administration of bradykinin: effects of Hoe 140. The effects of bradykinin were determined before, and 1 h, 2 h, 3 h and 4 h after bolus administration of (a) vehicle, (b) 0.01 mg kg^{-1} Hoe 140, (c) 0.1 mg kg^{-1} Hoe 140, and (d) 1.0 mg kg^{-1} Hoe 140. For clarity only the pretreatment (○) and the 1 h (□) and 4 h (△) dose-response curves are shown. Each point represents the mean $\pm$ s.e.mean of 4 observations.

(2) Renal effects of inhibition of ACE, EP 24.11 and EP 24.15

Resting haemodynamic and renal variables and the effects of Hoe 140 These are shown in Table 1. Levels of mean arterial pressure, heart rate, haematocrit and renal blood flow were within the normal range that we have observed previously using an almost identical infusion protocol, in rabbits with both kidneys intact (Evans *et al.*, 1994). In contrast, levels of glomerular filtration rate, filtration fraction and urine flow were about half those seen in two kidney rabbits, while levels of Na^+ excretion and fractional Na^+ excretion were about two fold and four fold elevated respectively. Fractional Na^+ reabsorption by the proximal tubules was also decreased in these one-kidney rabbits (24%) compared to our previous observations in two-kidney rabbits (62%) (Evans *et al.*, 1994). In the absence of any other treatment, the levels of these variables remained stable over the 80 min of the experiment (Figures 2 and 3).

Hoe 140 (0.1 mg kg^{-1}) had no effect on systemic or renal haemodynamic variables, but did significantly increase urine flow and the fractional excretion of Na^+. This natriuresis after Hoe 140 was associated with decreased fractional Na^+ reabsorption in the proximal tubules, but not the distal tubules (Table 1).

Effects of SCH 39370 and captopril Administration of captopril and SCH 39370 was followed by changes in systemic haemodynamic, renal haemodynamic and renal excretory variables that were maintained for the remaining three 20 min clearance periods (Figures 2 and 3). Mean arterial pressure fell by an average of 6 $\pm$ 2 mmHg and heart rate rose by an average of 35 $\pm$ 7 beats min^{-1}. Renal blood flow rose only slightly (0.7 $\pm$ 0.6 ml min^{-1} kg^{-1}), but when compared with vehicle-treatment, where renal blood flow decreased slightly, this effect was statistically significant (relative difference 1.2 $\pm$ 0.6 ml min^{-1} kg^{-1}, $P = 0.004$). Neither glomerular filtration rate nor filtration fraction (data not shown) were altered following administration of captopril and SCH 39370, but Na^+ excretion and fractional Na^+ excretion rose by an average of 14.3 $\pm$ 2.4 μmol min^{-1} kg^{-1} and 5.3 $\pm$ 1.0% respectively. Fractional reabsorption of Na^+ by both the proximal and distal tubules also decreased following captopril and SCH 39370 administration by 23.0 $\pm$ 2.5% and 3.8 $\pm$ 0.7%, respectively. Indeed, Na^+ reabsorption by the proximal tubules was virtually abolished after combined inhibition of ACE and EP 24.11, averaging -4.2 ± 5.9%.

Table 1 Baseline levels of haemodynamic and renal function variables in conscious rabbits: effects of Hoe 140 (0.1 mg kg^{-1})

Treatment	*Vehicle*	*Hoe 140*	P
MAP (mmHg)	84±2	86±2	0.52
HR (beats min^{-1})	236±9	238±17	0.92
Hct (%)	36.5±0.6	35.9±0.8	0.42
RBF (ml kg^{-1} min^{-1})	18.4±3.2	21.6±1.4	0.33
GFR (ml kg^{-1} min^{-1})	1.79±0.11	1.78±0.25	0.95
Filtration fraction (%)	19.9±3.1	13.3±1.9	0.13
Urine flow (ml kg^{-1} min^{-1})	0.077±0.020	0.156±0.027	0.01
$U_{Na}V$ (μmol kg^{-1} min^{-1})	9.8±1.2	13.3±1.0	0.10
%NA excretion (%)	4.2±0.7	6.0±1.2	0.05
FPR_{Na}(%)	24.0±2.9	10.5±4.3	0.001
FDR_{Na}(%)	94.6±0.7	93.4±1.1	0.25

Each value is the between-rabbit mean±s.e.mean of average values during the first 20 min clearance period in two-three separate experiments for 6 rabbits. Hoe 140 or its vehicle were administered intravenously 90 min before the start of this clearance period. MAP, mean arterial pressure; HR, heart rate; Hct, haematocrit; RBF, renal blood flow; GFR, glomerular filtration rate; $U_{Na}V$, urinary Na^+ excretion; %Na, fractional excretion of Na^+; FPR_{Na}, fractional proximal tubular reabsorption of Na^+; FDR_{Na}, fractional distal tubular reabsorption of Na^+. *P* values were derived from analysis of variance (d.f. = 1,22) testing for differences between levels in the absence and presence of Hoe 140.

When captopril and SCH 39370 were administered following treatment with Hoe 140, renal blood flow, Na^+ excretion, and the fractional excretion of Na^+ rose similarly to that seen in vehicle-pretreated rabbits (see above). Fractional Na^+ reabsorption by the proximal and distal tubules also decreased similarly to that seen in the absence of Hoe 140. However, when captopril and SCH 39370 were administered following Hoe 140-treatment, mean arterial pressure and heart rate did not change significantly (Figures 2 and 3).

Effects of cFP-AAY-pAB When cFP-AAY-pAB was administered on a background of inhibition of ACE and EP 24.11, no changes in systemic haemodynamic, renal haemodynamic or renal excretory variables were observed, regardless of whether the rabbits had been pretreated with Hoe 140 or its vehicle (Figures 4 and 5).

Plasma levels of SCH 39370 and cFP-AAY-pAB Plasma levels of cFP-AAY-pAB averaged 5.2±1.5 μM, 20 min after the start of cFP-AAY-pAB treatment. At the completion of the study, 40 min after cFP-AAY-pAB-treatment started, plasma levels averaged 2.9±0.9 μM. At this time, plasma levels of SCH 39370 averaged 5.4±0.6 μM.

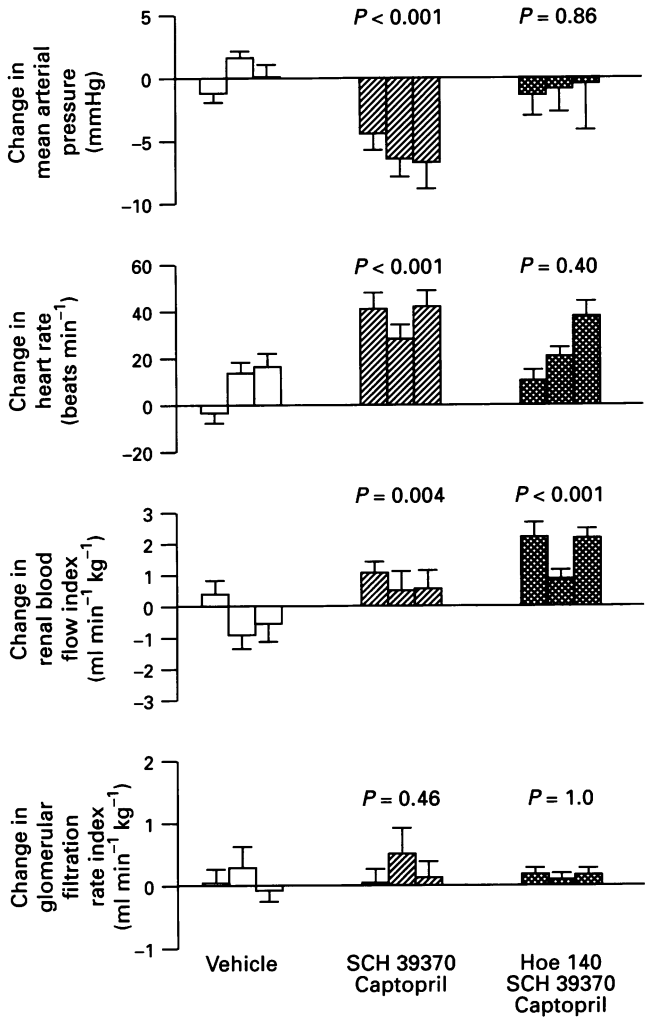

Figure 2 Changes in systemic and renal haemodynamic variables following combined treatment with the ACE inhibitor, captopril and the EP 24.11 inhibitor, SCH 39370, after pretreatment with the bradykinin antagonist Hoe 140 or its vehicle. Columns represent the mean changes from control levels (see Table 1) of each variable over 3 consecutive 20 min periods ($n = 6-7$). The treatments were: open columns, Hoe 140 vehicle followed by the captopril and SCH 39370 vehicle; hatched columns, Hoe 140 vehicle followed by captopril (0.25 mg kg^{-1} plus 0.2 mg kg^{-1} h^{-1}) and SCH 39370 (3 mg kg^{-1} plus 3 mg kg^{-1} h^{-1}); cross-hatched columns Hoe 140 (0.1 mg kg^{-1}) followed by captopril (0.25 mg kg^{-1} plus 0.2 mg kg^{-1} h^{-1}) and SCH 39370 (3 mg kg^{-1} plus 3 mg kg^{-1} h^{-1}). The *P* values represent the outcomes of two-factor analyses of variance (d.f. 1,24–27) testing for the main effect of inhibition of EP 24.11 and ACE. Thus, the hypotheses tested were whether the levels of each variable, averaged across experimental periods 2–4, changed differently in response to administration of captopril and SCH 39370; or Hoe 140, captopril and SCH 39370, compared with administration of vehicle alone. Because a total of 4 hypotheses were tested from this experiment (the other 2 are depicted in Figure 4), the *P* values were corrected by the Dunn-Sidak procedure where the corrected *P* value = $1-(1-P_x)^k$, where P_x = the raw *P* value and k = the number of multiple comparisons (4 in this case) (see Ludbrook, 1991).

Discussion

The results of the present study show that, at least under conditions where ACE and EP 24.11 are inhibited, EP 24.15 plays little or no role in modulating renal function in conscious rabbits. These experiments also provide additional information about the haemodynamic and renal effects of combined inhibiton of ACE and EP 24.11 in conscious rabbits, and about the *in vivo* pharmacology of the bradykinin B_2-receptor antagonist, Hoe 140.

Effects of inhibition of EP 24.15

We found that administration of the EP 24.15 inhibitor cFP-AAY-pAB (5 mg kg^{-1} plus 3 mg kg^{-1} h^{-1}), to rabbits pretreated with the ACE inhibitor, captopril and the EP 24.11 inhibitor, SCH 39370, did not alter any of the systemic haemodynamic or renal function variables we monitored. It was not possible for us to test directly whether EP 24.15 was inhibited *in vivo* by the dose of cFP-AAY-pAB administered in the present study, because there is at present no reliable bioassay in the intact animal for EP 24.15 activity. We have previously found that cFP-AAY-pAB does inhibit systemic bradykinin metabolism, augment the depressor effect of exogenous bradykinin, inhibit the pressor response to exogenous angiotensin I, and lower basal arterial pressure in conscious

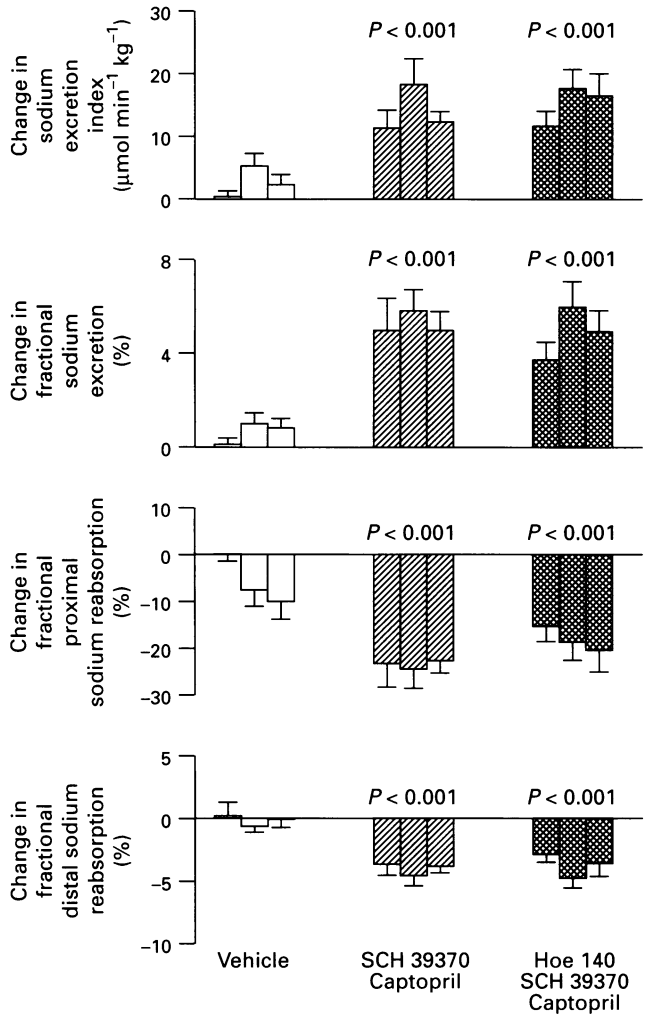

Figure 3 Changes in renal Na^+ handling variables following combined treatment with the ACE inhibitor captopril and the EP 24.11 inhibitor SCH 39370, after pretreatment with the bradykinin antagonist, Hoe 140 or its vehicle. Columns represent the mean changes from control levels (see Table 1) of each variable over 3 consecutive 20 min periods ($n = 6-7$). The treatments and P values are the same as for Figure 2.

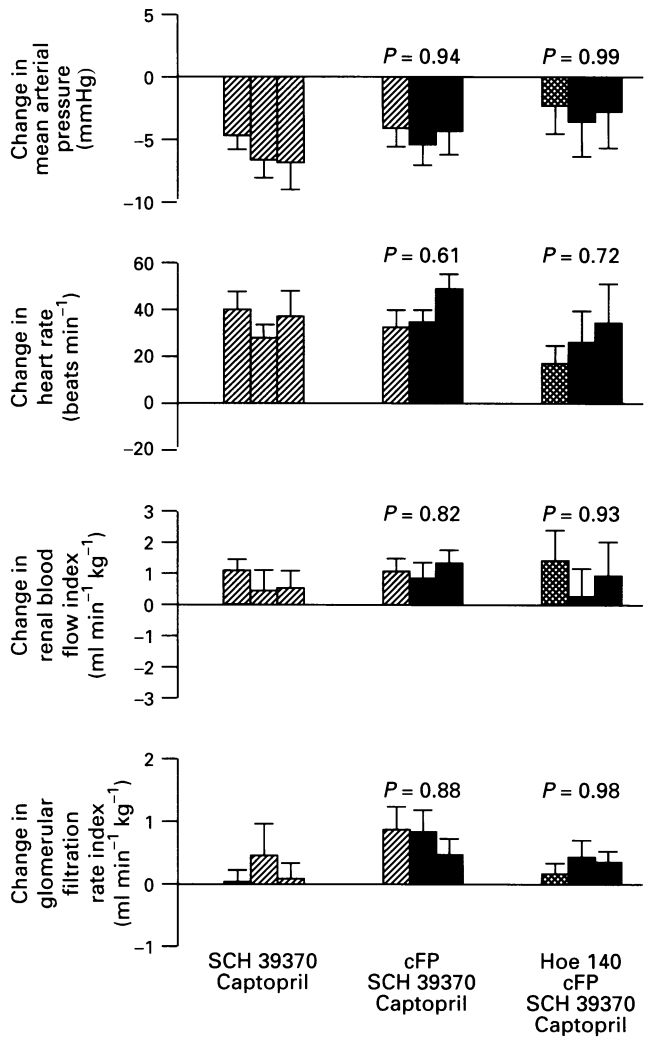

Figure 4 Changes in systemic and renal haemodynamic variables following treatment with the EP 24.15 inhibitor cFP-AAY-pAB, in rabbits pretreated with a combination of the ACE inhibitor captopril and the EP 24.11 inhibitor, SCH 39370 and either the bradykinin antagonist Hoe 140 or its vehicle. Columns represent the mean changes from control levels (see Table 1) of each variable over 3 consecutive 20 min periods ($n = 6-7$). The treatments were: hatched columns, Hoe 140 vehicle followed by captopril (0.25 mg kg^{-1} plus 0.2 mg kg^{-1} h^{-1}) and SCH 39370 (3 mg kg^{-1} plus 3 mg kg^{-1} h^{-1}); cross-hatched columns, Hoe 140 (0.1 mg kg^{-1}) followed by captopril (0.25 mg kg^{-1} plus 0.2 mg kg^{-1} h^{-1}) and SCH 39370 (3 mg kg^{-1} plus 3 mg kg^{-1} h^{-1}); solid columns, cFP-AAY-pAB (5 mg kg^{-1} plus 3 mg kg^{-1} h^{-1}). The P values represent the outcomes of repeated-measures analyses of variance (d.f. 2,22-24) testing for non-parallelism between cFP-AAY-pAB treatment and administration of its vehicle (treatment × time interaction). Thus, the hypotheses tested were whether the levels of the variables changed differently in response to cFP-AAY-pAB administration compared with administration of its vehicle. Note that these hypotheses are different from those tested in Figures 2 and 3. Because a total of 4 hypotheses were tested from this experiment (the other 2 are depicted in Figure 2), the P values were corrected by the Dunn-Sidak procedure as for Figure 2.

rabbits. However, these effects are entirely attributable to inhibition of ACE by the metabolite cFP-AA (Telford *et al.*, 1995; Lew *et al.*, 1996). Similar observations have been made in anaesthetized rats in which the phenylalanine substituted analogue cFP-AAF-pAB was used (Yang *et al.*, 1994). We can, however, be confident that the dose of cFP-AAY-pAB administered was sufficient to inhibit EP 24.15, since its plasma levels were orders of magnitude above its K_i for EP 24.15 (16 nM; Orlowski *et al.*, 1988).

In terms of effects on renal function, our observations contrast with those of Yang *et al.* (1994), who found that in rats pretreated with only the ACE inhibitor enalaprilat, cFP-AAF-pAB increased glomerular filtration rate, urine volume, and the fractional excretion of Na^+. There are a number of possible reasons for this discrepancy, including differences in experimental conditions between the two studies. Thus, Yang *et al.* (1994) studied anaesthetized rats with intact kidneys, while we used conscious uninephrectomized rabbits, in which renal function (particularly proximal tubular Na^+ reabsorption) had changed to compensate for the reduced renal mass. Yang *et al.* (1994) concluded from their study that 'cFP-AAF-pAB might act on metalloendopeptidase 24.15 or peptidases other than ACE to inhibit degradation of vasoactive peptides that participate in the regulation of renal function'. We suggest, based on the results of our study, that a likely site of action of cFP-AAF-pAB in their experiment was EP 24.11 rather than EP 24.15. Although both cFP-AAY-pAB and cFP-AAF-pAB have low affinity for EP 24.11, they can inhibit this enzyme at higher concentrations (K_i values of 35 and 17 μM respectively). We have found previous in studies in conscious rabbits that a bolus dose of cFP-AAY-pAB of 5 mg kg^{-1}, although rapidly cleared from the circulation (100 fold lower levels in 5 min), results in peak blood levels over 100 μM (Lew *et al.*, 1996), which would be expected to inhibit EP 24.11. The dose of cFP-AAF-pAB administered by Yang *et al.* (1994) was

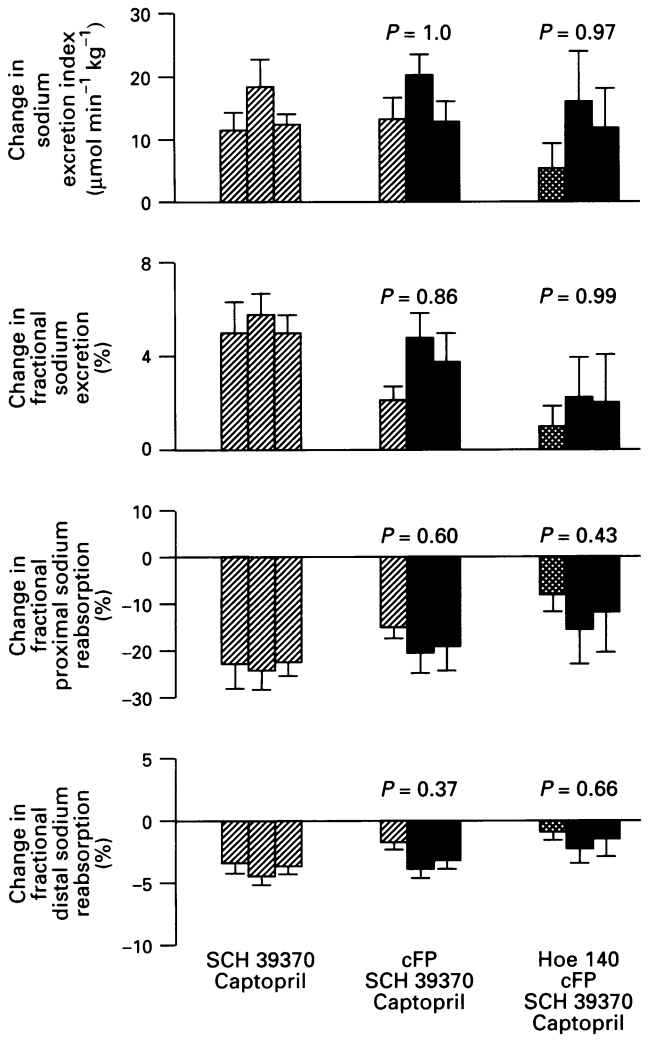

Figure 5 Changes in renal Na^+ handling variables following treatment with the EP 24.15 inhibitor cFP-AAY-pAB, in rabbits pretreated with a combination of the ACE inhibitor, captopril and the EP 24.11 inhibitor, SCH 39370 and either the bradykinin antagonist Hoe 140 or its vehicle. Columns represent the mean changes from control levels (see Table 1) of each variable over 3 consecutive 20 min periods ($n=6-7$). The treatments and P values are the same as for Figure 4.

similar to ours, so would probably have also resulted in peak blood levels sufficient to block EP 24.11. Certainly, in the present study in which cFP-AAY-pAB was administered as both a bolus and a slow infusion, blood levels remained sufficient to at least partially inhibit EP 24.11 (2.9–5.2 μM). We therefore conclude that on balance the evidence suggests that, at least under conditions where ACE and EP 24.11 are already inhibited, EP 24.15 plays little or no role in regulating renal function, and that the renal effects of cFP-AAF-pAB seen previously by Yang *et al.* (1994) are probably attributable to inhibition of EP 24.11.

Effects of Hoe 140 in conscious rabbits

Hoe 140 is a selective and potent non-competitive antagonist at bradykinin B_2 receptors (Wirth *et al.*, 1991; Rhaleb *et al.*, 1992). In some experimental situations this compound does exhibit partial agonist activity (Wirth *et al.*, 1991; Félétou *et al.*, 1994), so we performed a preliminary experiment to determine a dose of Hoe 140 that would antagonize the cardiovascular effects of exogenous bradykinin over the period of a few hours, without exerting bradykinin-like haemodynamic effects of its own. We found that doses of Hoe 140 as low as 10 μg kg^{-1} completely abolished the depressor response to 1 μg kg^{-1} bradykinin, which in untreated animals transiently lowered mean arterial pressure by about 35 mmHg. Small depressor responses to bradykinin (10–100 μg kg^{-1}) were seen after 10 μg kg^{-1} Hoe 140, particularly 3–4 h after the antagonist was administered. Higher doses of Hoe 140 (0.1–1.0 mg kg^{-1}) antagonized the effects of bradykinin more completely, with no recovery of the effect of bradykinin being seen over a 4 h period. None of the doses of Hoe 140 had any acute effect on arterial pressure or heart rate.

We chose to use a dose of 0.1 mg kg^{-1} Hoe 140 in our subsequent experiment, and unexpectedly found that it increased Na^+ excretion. We conclude that this natriuretic effect was mediated predominantly by inhibition of proximal tubular Na^+ reabsorption, since our estimate of fractional proximal- but not fractional distal-tubular Na^+ reabsorption was significantly decreased. These effects are unlikely to reflect antagonism of the effects of endogenous bradykinin, since intrarenally formed bradykinin most likely inhibits predominantly distal tubular and collecting duct Na^+ reabsorption. Thus, although B_2-receptors are present in the proximal tubule, the cellular machinery for bradykinin synthesis is mainly confined to the distal tubule (see Figueroa *et al.*, 1995; Majima & Katori, 1995; Saitoh *et al.*, 1995). The renal effects of Hoe 140 more likely reflect its partial agonist property. This hypothesis is supported by the fact that the effects of Hoe 140 we observed, closely resemble those of intrarenally infused bradykinin (see Willis *et al.*, 1969; Ader *et al.*, 1992).

Effects of combined inhibition of ACE and EP 24.11 in conscious rabbits

Recently there has been increased interest in the possible therapeutic efficacy of dual inhibition of ACE and EP 24.11 in the treatment of hypertension. In the present study we tested the effects of dual inhibition of these two enzyme systems in conscious rabbits. We can be confident that the dose regimens we used provided profound inhibition of these two enzyme systems. In the case of captopril, we have shown previously that the regimen we used abolishes the haemodynamic response to intravenous administration of angiotensin I (10–100 ng kg^{-1}) and profoundly inhibits the systemic degradation of exogenous bradykinin (Telford *et al.*, 1995). In the case of SCH 39370, we measured blood levels of this compound at the time of termination of each study, and found them to average about 5 μM; orders of magnitude greater than the K_i of this inhibitor for EP 24.11 (5–11 nM; Sybertz *et al.*, 1989).

We found that combined administration of captopril and SCH 39370 reduced arterial pressure and increased heart rate, increased renal blood flow without changes in glomerular filtration rate, and increased both the absolute and fractional excretion of Na^+. Inhibition of both proximal and distal tubular Na^+ reabsorption appeared to mediate the natriuretic effect of inhibition of these enzymes. These observations are consistent with those in other species. In conscious rats (both normotensive and hypertensive) (Pham *et al.*, 1993; French *et al.*, 1995; Vera *et al.*, 1995) and in conscious dogs with pacing-induced heart failure (Seymour *et al.*, 1993), dual inhibition of both enzyme systems is followed by falls in mean arterial pressure. In contrast, in healthy normotensive men, mean arterial pressure appears to be unaffected (Motwani *et al.*, 1995). In all of these experimental models, however, increases in urine volume and Na^+ excretion have been observed, and when it has been tested, increases in renal blood flow without changes in glomerular filtration rate (Motwani *et al.*, 1995).

Combined inhibition of ACE and EP 24.11 also consistently increases urinary excretion of cyclic guanosine monophosphate, atrial natriuretic peptide and bradykinin (Pham *et al.*, 1993; Seymour *et al.*, 1993; French *et al.*, 1995; Vera *et al.*, 1995). These observations suggest that inhibition of both atrial natriuretic peptide and bradykinin metabolism within the kidney as well as suppression of the renin angiotensin system, might contribute to the renal effects of combined inhibition of

these enzymes. This hypothesis, however, has not yet been formally tested. There is also evidence from studies with bradykinin antagonists suggesting that bradykinin can contribute to the renal effects of ACE inhibition alone (Kon *et al.*, 1993; Bouaziz *et al.*, 1994) and EP 24.11 inhibition alone (Ura *et al.*, 1994). We tested the effects of the bradykinin B_2-receptor antagonist Hoe 140 on the systemic haemodynamic and renal effects of combined inhibition of ACE and EP 24.11. We found that Hoe 140 had no effect on the renal actions of combined treatment with captopril and SCH 39370, but did prevent the reduction in arterial pressure and rise in heart rate. This observation is somewhat confounded by the apparent partial agonist activity of Hoe 140 which is exerted on the kidney under the conditions of our experiment; but it does give a clear indication that increased renal levels of bradykinin do not make a major contribution to the renal effects of combined inhibition of ACE and EP 24.11 observed under the present experimental conditions. These observations are consistent with those of Chen & Zimmerman (1994) who found in anaesthetized rabbits that the renal haemodynamic effects of ACE inhibition were not affected by Hoe 140.

Our results also suggest that increased levels of endogenous bradykinin do contribute to the depressor effects of combined inhibition of ACE and EP 24.11. These observations are consistent with a recent study showing that Hoe 140 blunts the blood pressure lowering effects of ACE inhibition in spontaneously hypertensive rats (Bouaziz *et al.*, 1994). Because we did not test the effects of Hoe 140 on the systemic haemodynamic effects of SCH 39370 and captopril given alone, we cannot say with any certainty whether the hypotension we observed was due to inhibition of ACE, EP 24.11, or the combined effects of inhibition of both enzymes. However, since the hypotension we observed appears to be chiefly attributable to the influence of endogenous bradykinin, and since we have previously found that captopril but not SCH 39370 potentiates the hypotensive effect of bradykinin (Telford *et al.*, 1995; Tomoda *et al.*, 1995; Lew *et al.*, 1996), it seems likely that inhibition of EP 24.11 contributed minimally to the hypotensive effect of combined treatment with captopril and SCH 39370.

Conclusions

In a previous study, we demonstrated that although cFP-AAY-pAB (5 mg kg^{-1}) inhibited systemic bradykinin metabolism in conscious rabbits, this effect was entirely attributable to inhibition of ACE (Telford *et al.*, 1995). More recently we have demonstrated that this dose of cFP-AAY-pAB was sufficient to inhibit EP 24.15 (Lew *et al.*, 1996), so can confidently conclude that EP 24.15 plays little or no role in systemic bradykinin metabolism in conscious rabbits. The results of the present study suggest that EP 24.15 does not play a major role in the metabolism of vasoactive peptides which modulate renal function in conscious rabbits, since at least when ACE and EP 24.11 are blocked, cFP-AAY-pAB does not affect renal function. The high levels of EP 24.15 found in the brain, pituitary and testis (Chu & Orlowski, 1985) suggest that these tissues might be more likely sites for the physiological roles of EP 24.15 in peptide metabolism. The execution and interpretation of experiments aimed at elucidating these roles will be greatly simplified if potent and selective non-peptide inhibitors of EP 24.15 can be developed.

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