Response to atrial natriuretic peptide, endopeptidase 24.11 inhibitor and C-ANP receptor ligand in the rat

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1 The present studies compared the renal and hypotensive response to (a) exogenous atrial natriuretic peptide (ANP) (99–126), (b) an endopeptidase-24.11 inhibitor (candoxatrilat) and (c) an antagonist of ANP clearance receptors (SC 46542) in conscious rats.

2 Infusion of low-dose-ANP ($100 \text{ ng kg}^{-1} \text{ min}^{-1}$) produced a gradual increase in urinary sodium and guanosine 3':5'-cyclic monophosphate (cyclic GMP) excretion without significant change in glomerular filtration rate (GFR) or fractional lithium clearance (FeLi). There was a significant fall in blood pressure.

3 Infusion of high-dose ANP ($300 \text{ ng kg}^{-1} \text{ min}^{-1}$) produced a brisk, 3 fold increase in urinary sodium and cyclic GMP excretion along with a rise in GFR, but had no significant effect on FeLi compared to the control group. The renal response was accompanied by a pronounced fall in blood pressure.

4 Candoxatrilat or SC 46542, alone, had no significant effect on sodium excretion compared to control animals. Both compounds enhanced the natriuretic and cyclic GMP responses to a low-dose ANP infusion ($100 \text{ ng kg}^{-1} \text{ min}^{-1}$) to levels similar to, or greater than, those observed with the high-dose ANP ($300 \text{ ng kg}^{-1} \text{ min}^{-1}$). However, unlike high-dose ANP, these renal effects were not accompanied by a significant change in GFR and neither compound potentiated the hypotensive effect of the low-dose ANP infusion. Only candoxatrilat when given with ANP produced a marked rise in FeLi.

5 Similarly, combined administration of candoxatrilat and SC 46542 (without exogenous ANP) induced an increase in sodium and cyclic GMP excretion comparable to high-dose ANP but did so without a significant increase in GFR and with a significantly smaller fall in blood pressure. Interestingly, there was no increase in FeLi with the combination of the two compounds, suggesting that the major contribution to sodium excretion came from SC 46542.

6 Both candoxatrilat and SC 46542 increased sodium and cyclic GMP excretion in the rat A-V fistula model of heart failure, a model hyporesponsive to infusions of ANP, without significant change in blood pressure.

7 These data show that candoxatrilat and SC 46542 do not simply reproduce the effects of an ANP infusion but preferentially enhance the natriuretic response to ANP. Inhibition of E-24.11 may potentiate a tubule action of ANP while the renal mechanism of action of the C-ANP receptor ligand needs further study. Both manipulations are of potential value in the management of heart failure.

Keywords: Atrial natriuretic peptide; lithium clearance; cardiac failure

Introduction

The profile of biological activity of atrial natriuretic peptide (ANP 99–126) – natriuresis-diuresis, vasorelaxation and inhibition of the renin-angiotensin system – has led to interest in the therapeutic potential of this peptide in cardiovascular diseases such as heart failure and hypertension (Brenner *et al.*, 1990). Use of the peptide itself as a drug is limited by its short plasma half-life (2–3 min) and its poor oral bioavailability. Instead, attention has focused on enhancing the activity of endogenous ANP, in particular, by inhibiting its clearance.

In addition to filtration at the glomerulus, two mechanisms have been shown to contribute to the clearance of ANP, namely, metabolism by endopeptidase-24.11 (E-24.11) and internalisation via clearance (C-ANP) receptors. E-24.11 is an ectoenzyme with a wide tissue distribution that includes the renal brush border, brain and intestine (Gee *et al.*, 1985; Matsas *et al.*, 1986). The enzyme cleaves ANP at position Cys¹⁰⁵-Phe¹⁰⁶ in the peptide ring to yield an inactive metabolite (Olins *et al.*, 1989). E-24.11 inhibitors prolong the half-life of co-administered ANP *in vivo* and enhance its natriuretic effect (Trapani *et al.*, 1989; Barclay *et al.*, 1991).

ANP receptors can be divided into two major groups; A-ANP and B-ANP receptors are coupled to guanylate cyclase (Chinkers *et al.*, 1989; Koller *et al.*, 1991), while C-ANP receptors are not linked to this enzyme (Porter *et al.*, 1990). A-ANP receptors are thought to mediate the biological actions of ANP. B-ANP receptors may be responsible for the actions of another member of the natriuretic peptide family, known as C-natriuretic peptide (Koller *et al.*, 1991). C-ANP receptors, on the other hand, have been shown to internalise and degrade ANP (Nussenzveig *et al.*, 1990). Widely distributed in a number of tissues (for example, vascular smooth muscle and endothelial cells and renal glomerulus) C-ANP receptors account for the majority of ANP binding sites and are thought to perform a clearance role (Maack *et al.*, 1987). In support of this, specific C-ANP receptor ligands exhibit no intrinsic natriuretic-diuretic activity in the isolated kidney but increase plasma ANP levels and stimulate a natriuresis when infused *in vivo* (Suzuki *et al.*, 1987; Almeida *et al.*, 1989).

If E-24.11 inhibitors and C-ANP receptor ligands act simply to increase plasma ANP levels by reducing the clearance of endogenous ANP, then these compounds should reproduce the effects of an ANP infusion. Moreover, comparison of the effect of C-ANP receptor ligand and E-24.11 inhibitor on the pharmacokinetics of [¹²⁵I]-ANP suggests that the C-ANP pathway has the greater capacity for clearing circulating ANP (Chiu *et al.*, 1991). It might be predicted

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therefore that C-ANP receptor ligands would be more useful than E-24.11 inhibitors in potentiating the actions of endogenous ANP. However, studies in animal models of heart failure and hypertension show that the renal response to E-24.11 inhibitors does not correlate with their effects on plasma ANP concentration (Wilkins *et al.*, 1990b; Seymour *et al.*, 1991). It has been suggested that the efficacy of E-24.11 inhibitors in heart failure derives, at least in part, from potentiation of the renal tubule actions of ANP (Wilkins *et al.*, 1990b). This concept supported by the observation that the E-24.11 inhibitor, SQ 28603, increases fractional lithium excretion (Margulies *et al.*, 1990), a marker of pre-distal tubule sodium reabsorption. The extent to which this effect is shared by C-ANP receptor ligands is not known.

Accordingly, the present studies compared the renal and hypotensive responses to manipulation of ANP activity with an E-24.11 inhibitor (candoxatrilat) or a C-ANP receptor ligand (SC 46542) with infusion of exogenous ANP in the conscious rat. To assess the therapeutic potential of these manipulations, the effects of candoxatrilat and SC 46542 were also examined in rats with aorto-caval (A-V) fistulae, a model of heart failure with chronically elevated endogenous ANP levels but hyporesponsive to the effects of an ANP infusion (Wilkins *et al.*, 1990b).

Methods

Infusion protocol

Studies were performed on male Wistar rats (250–300 g). Under Hypnorm (fentanyl/fluanisone) anaesthesia, both femoral veins, a femoral artery and the bladder were cannulated with portex tubing (0.58 mm). The animals were allowed to regain consciousness in individual restraining cages. An infusion of sodium chloride 150 mM (0.5 ml h⁻¹) containing lithium chloride 25 mM and [³H]-inulin (Amersham) 3 µCi ml⁻¹ was given via one femoral vein and continued for the study duration. A second infusion of sodium chloride 150 mM (1 ml h⁻¹) was given via the other femoral vein and used for administration of drugs. Observations were started 2.5 h after beginning these infusions; while still in the acute recovery phase post-surgery, glomerular filtration rate (GFR) and urine output are sufficiently stable to provide a background against which responses can be measured. Urine was collected at 15 min intervals into preweighed tubes for determination of volume, sodium, potassium, lithium, guanosine 3':5'-cyclic monophosphate (cyclic GMP) and [³H]-inulin concentrations. Blood pressure was measured via the femoral artery every 15 min (MacLab instruments). Arterial blood samples (200 µl) were taken into haematocrit tubes 30 and 90 min from the start of observations for lithium and [³H]-inulin measurement.

Rat ANP (99–126) was purchased from Bachem, U.K. Candoxatrilat is a specific E-24.11 inhibitor (Danilewicz *et al.*, 1989) and was a gift from Pfizer UK. SC 46542 is des(Phe¹⁰⁶Gly¹⁰⁷Ala¹¹⁵Gln¹¹⁶)-ANP(5–28) and was synthesized by Monsanto Company, St Louis, U.S.A.; it binds specifically to C-ANP receptors (Koepeke *et al.*, 1989).

Studies in normal rats

ANP infusions To examine the response of rats to ANP, animals received exogenous ANP (99–126) 100 ng kg⁻¹ min⁻¹, 300 ng kg⁻¹ min⁻¹ or vehicle (saline) alone as a 60 min infusion (*n* = 6 each group). In a separate set of experiments, animals were set up as per protocol but blood (2 ml) was collected at the end of an infusion of ANP 100 ng kg⁻¹ min⁻¹ into potassium EDTA (2 mg ml⁻¹) tubes on ice for plasma ANP assay.

Studies with candoxatrilat and SC 46542 To investigate the synergistic effect of inhibition of E-24.11 and co-infusion of

ANP, candoxatrilat was given in doses of 1, 3, or 10 mg kg⁻¹ (*n* = 6 to 9 each group) by bolus injection over 1 min at the start of a 60 min infusion of ANP 100 ng kg⁻¹ min⁻¹. Control animals received candoxatrilat 10 mg kg⁻¹ alone (*n* = 6). Doses of candoxatrilat ≥ 3 mg kg⁻¹ have been shown to have a maximal natriuretic effect in hydrated rats (Shepperson *et al.*, 1991).

To investigate the effect of a C-ANP receptor ligand on the response to ANP, two doses of SC 46542 (68 µg kg⁻¹ bolus; followed by 6.8 µg kg⁻¹ min⁻¹ for 60 min (*n* = 9); or 680 µg kg⁻¹ bolus; then 68 µg kg⁻¹ min⁻¹ for 60 min (*n* = 6)) were given with a 60 min infusion of ANP (100 ng kg⁻¹ min⁻¹). A control group received the higher dose of SC 46542 alone (*n* = 6).

The effect of combined inhibition of both clearance mechanisms in the absence of exogenous ANP was examined by giving candoxatrilat 3 mg kg⁻¹ by bolus injection before the higher dose of SC 46542 (680 µg kg⁻¹ bolus, then 68 µg kg⁻¹ min⁻¹ for 60 min (*n* = 6)).

Studies in A-V fistula rats A-V fistula surgery was performed under Hypnorm anaesthesia. The fistula (1–1.5 mm long) was made through a side to side anastomosis between aorta and inferior vena cava approximately 10 mm distal to the renal arteries. Sham-operations were performed by exposing and temporarily clamping (5 min) the aorta and vena cava without cutting or suturing the vessels. After surgery, the animals were returned to their cages and allowed free access to water and a standard rat diet. The rats were studied 10 to 14 days post-surgery. All A-V fistula and sham-operated rats were placed in metabolic cages for 24 h before study for urine collection for cyclic GMP assay. To determine the levels of circulating ANP achieved in this model, A-V fistula and sham-operated animals (*n* = 8 each group) were randomly selected for blood sampling only; carotid artery and femoral vein cannulae were implanted and the animals allowed to recover in restraining cages for 2.5 to 3 h before blood sample collection. Blood (2 ml) was collected into potassium EDTA (2 mg ml⁻¹) tubes on ice. Those animals not used for blood sampling were used for further study. The femoral veins, carotid artery and bladder were cannulated and the infusion protocol described above was used with the exception that lithium was omitted from the infusate. The A-V fistula rats were divided into the following treatment groups: (a) candoxatrilat 3 mg kg⁻¹ (*n* = 7) by bolus injection; (b) SC 46542 68 µg kg⁻¹ bolus; followed by 6.8 µg kg⁻¹ min⁻¹ for 60 min, (*n* = 6); (c) SC 46542 680 µg kg⁻¹ bolus; followed by 68 µg kg⁻¹ min⁻¹ for 60 min (*n* = 7); (d) a combination of (a) and (c) (*n* = 4); and (e) vehicle alone (*n* = 6). Sham-operated animals received treatment (a) or (c) with 6 animals in each group. At the end of the experiment the hearts were removed and the weights recorded.

Assays

Urine volume was measured gravimetrically. Sodium concentration was measured by flame photometry (Corning 480) and lithium by atomic absorption spectroscopy. [³H]-inulin levels were determined by liquid scintillation counting in Insta-gel (Packard). Urinary cyclic GMP concentration was measured by radioimmunoassay on appropriately diluted samples as previously described (Wilkins *et al.*, 1990a). Plasma ANP concentrations were measured on unextracted samples with a radioimmunoassay kit (Peninsula Laboratories).

Statistics

Data are presented as mean ± s.e.mean. Blood pressure and renal responses were examined by one way analysis of variance with respect to treatment using time (urine collection) as a covariate. Differences were assessed for statistical significance using least significance difference test. Heart

weight and 24 h urinary cyclic GMP excretion differences were assessed by Students *t* test, paired or unpaired, as appropriate. All calculations were made with Complete Statistical System (StatSoft) software. Statistical significance was assumed when the *P* value was <0.05 .

Results

ANP infusions in normal rats

Given alone, ANP $100 \text{ ng kg}^{-1} \text{ min}^{-1}$ produced a small increase in urinary sodium and cyclic GMP excretion compared to vehicle treated controls (Figure 1). In the subgroup of rats killed for plasma sampling, the mean plasma ANP level at the end of the ANP infusion was $533 \pm 68 \text{ pg ml}^{-1}$ compared with $102 \pm 33 \text{ pg ml}^{-1}$ in the control group ($P < 0.05$).

Increasing the dose of ANP to $300 \text{ ng kg}^{-1} \text{ min}^{-1}$ caused a rapid 5 fold rise in sodium output, and a near 8 fold rise in urinary cyclic GMP excretion; both changes were significantly greater than the effect of ANP $100 \text{ ng kg}^{-1} \text{ min}^{-1}$ (Figure 1). The early natriuresis (first 30 min) was accompanied by a significant increase in GFR ($P < 0.05$) compared to the vehicle control group, which was not seen with the lower dose of peptide (Table 1). Neither dose of ANP significantly affected fractional lithium excretion (FeLi) when compared to the control group.

Both doses of ANP reduced mean arterial blood pressure compared to vehicle-treated rats (Table 1). The fall in blood pressure with ANP $300 \text{ ng kg}^{-1} \text{ min}^{-1}$ was significantly greater than that produced by $100 \text{ ng kg}^{-1} \text{ min}^{-1}$.

Studies with candoxatrilat and SC 46542 in normal rats

Candoxatrilat on its own (in doses up to 10 mg kg^{-1}) had no significant effect on sodium excretion compared to vehicle-treated controls, but clearly enhanced the natriuretic response to co-infused ANP (Figure 2). Bolus injection of candoxatrilat 3 or 10 mg kg^{-1} before ANP $100 \text{ ng kg}^{-1} \text{ min}^{-1}$ produced a prompt 5 to 6 fold rise in sodium excretion within 30 min of beginning the ANP infusion. The response to candoxatrilat 1 mg kg^{-1} and ANP was similar in magnitude, but the peak effect was delayed (45 min). Thus 3 mg kg^{-1} was taken as representative of the top of the dose-response curve for the E-24.11 inhibitor. Consistent with potentiation of ANP, the increases in sodium excretion were accompanied by marked rises in urinary cyclic GMP excretion.

Similarly, a small, but non-significant, rise in sodium ex-

Figure 1 Effect of two doses of atrial natriuretic peptide (ANP, $100 \text{ ng kg}^{-1} \text{ min}^{-1}$, ■, and $300 \text{ ng kg}^{-1} \text{ min}^{-1}$, □) and vehicle (○) alone by infusion on urinary sodium (Na^+) excretion, cyclic GMP excretion and fractional lithium (Li^+) excretion in conscious normotensive rats. Saline ($100 \mu\text{l}$) was given prior to infusion as a control for subsequent experiments. Data are mean with s.e.mean shown by vertical bars. * $P < 0.05$ compared to time-controlled vehicle infusion group.

Table 1 Effect of atrial natriuretic peptide (ANP), E-24.11 inhibitor (Can) and C-ANP receptor ligand (SC46542) on mean blood pressure (BP) and glomerular filtration rate (GFR) in conscious normotensive rats

	Time (min)						
	15	30	45	60	75	90	120
BP (mmHg)							
Saline	110 ± 4	109 ± 3	111 ± 4	112 ± 4	109 ± 3	110 ± 4	111 ± 4
ANP $100 \text{ ng kg}^{-1} \text{ min}^{-1}$	108 ± 4	105 ± 4	103 ± 3	99 ± 3*	97 ± 2*	96 ± 4*	102 ± 5
ANP $300 \text{ ng kg}^{-1} \text{ min}^{-1}$	109 ± 3	107 ± 4	96 ± 3*	91 ± 3*	85 ± 1*†	84 ± 2*†	87 ± 2*†
ANP + Can 3 mg kg^{-1}	104 ± 5	107 ± 4	105 ± 4	100 ± 3*	96 ± 3*	96 ± 3*	99 ± 3*
ANP + SC 46542	107 ± 3	107 ± 3	102 ± 4	97 ± 3*	93 ± 2*	91 ± 4*	94 ± 4*
Can + SC 46542	107 ± 4	105 ± 4	107 ± 3	104 ± 3*	102 ± 3*	101 ± 4*	101 ± 4*
GFR (ml min⁻¹)							
Saline	3.2 ± 0.3	3.5 ± 0.4	3.5 ± 0.3	3.3 ± 0.3	3.6 ± 0.3	3.3 ± 0.3	3.1 ± 0.4
ANP $100 \text{ ng kg}^{-1} \text{ min}^{-1}$	3.4 ± 0.2	3.4 ± 0.2	3.1 ± 0.3	3.2 ± 0.3	3.3 ± 0.3	3.1 ± 0.3	2.5 ± 0.3
ANP $300 \text{ ng kg}^{-1} \text{ min}^{-1}$	3.3 ± 0.3	3.1 ± 0.2	4.5 ± 0.4†	4.2 ± 0.5†	3.5 ± 0.4	2.7 ± 0.3	2.9 ± 0.3
ANP + Can 3 mg kg^{-1}	3.0 ± 0.3	3.1 ± 0.3	3.7 ± 0.3	3.2 ± 0.2	3.0 ± 0.2	3.0 ± 0.2	2.8 ± 0.5
ANP + SC 46542	2.7 ± 0.2	3.0 ± 0.3	3.9 ± 0.4	2.9 ± 0.3	2.8 ± 0.5	2.6 ± 0.5	2.5 ± 0.1
Can + SC 46542	3.8 ± 0.4	3.6 ± 0.3	4.2 ± 0.3	3.6 ± 0.4	3.5 ± 0.3	3.4 ± 0.3	3.2 ± 0.2

Data are mean ± s.e.mean. Atrial natriuretic peptide (ANP) was infused from 30 to 90 min. Candoxatrilat (Can 3 mg kg^{-1}) was given prior to ANP $100 \text{ ng kg}^{-1} \text{ min}^{-1}$. SC 46542 was given as $680 \mu\text{g kg}^{-1}$ bolus then $68 \mu\text{g kg}^{-1} \text{ min}^{-1}$ during ANP $100 \text{ ng kg}^{-1} \text{ min}^{-1}$ or with candoxatrilat.

* $P < 0.05$ compared to saline; † $P < 0.05$ compared to ANP $100 \text{ ng kg}^{-1} \text{ min}^{-1}$.

Figure 2 Effect of candoxatrilat (3 mg kg^{-1} , ○), atrial natriuretic peptide (ANP) infusion ($100 \text{ ng kg}^{-1} \text{ min}^{-1}$, ■) and the combination (□) on urinary sodium (Na^+), cyclic GMP, and fractional lithium (Li^+) excretion in conscious normal rats. Data are mean with s.e.mean shown by vertical bars. * $P < 0.05$, ** $P < 0.001$, compared to ANP infusion alone group. (Data on response to co-treatment with candoxatrilat 1 and 10 mg kg^{-1} and ANP are omitted for clarity).

cretion was seen with SC 46542 alone, but when co-administered with low-dose ANP, SC 46542 caused a pronounced, dose-dependent natriuresis and rise in urinary cyclic GMP excretion (Figure 3). The effect of combining the higher dose of SC 46542 ($680 \mu\text{g kg}^{-1}$ bolus; $68 \mu\text{g kg}^{-1} \text{ min}^{-1}$) with low-dose ANP exceeded the maximal responses obtained with combined E-24.11 inhibition and ANP, although this did not reach statistical significance ($P = 0.059$).

An important difference between candoxatrilat and SC 46542, however, was observed in their effects of FeLi (Figures 2 and 3). The small, non-significant rise in FeLi seen with ANP alone during the course of the experiment was not significantly affected by the addition of SC 46542. In contrast, a marked and sustained rise in FeLi was obtained when candoxatrilat and ANP were given together.

The combination of candoxatrilat and SC 46542 (without exogenous ANP) produced an increase in urinary sodium and cyclic GMP excretion that was significantly ($P < 0.01$) greater than either agent alone (Figure 4); indeed, the peak response matched that produced by combining either of these compounds with a low-dose ANP infusion. Interestingly, the natriuretic response to combined inhibition of both clearance pathways was not associated with a significant rise in FeLi excretion.

The addition of candoxatrilat or SC 46542 to low-dose ANP and the co-administration of the two clearance inhibitors produced only transient rises in GFR in the first period

Figure 3 Effect of two doses of SC 46542 ($68 \mu\text{g kg}^{-1}$ bolus, then $6.8 \mu\text{g kg}^{-1} \text{ min}^{-1}$, Δ; or $680 \mu\text{g kg}^{-1}$ bolus, then $68 \mu\text{g kg}^{-1} \text{ min}^{-1}$, □) in combination with ANP ($100 \text{ ng kg}^{-1} \text{ min}^{-1}$, ■) on urinary sodium (Na^+), cyclic GMP and fractional lithium (Li^+) excretion in conscious rats. Controls received ANP ($100 \text{ ng kg}^{-1} \text{ min}^{-1}$, ■) or SC 46542 ($680 \mu\text{g kg}^{-1}$ bolus, $68 \mu\text{g kg}^{-1} \text{ min}^{-1}$, ○) alone. Data are mean with s.e.mean shown by vertical bars. * $P < 0.05$, ** $P < 0.01$ compared to ANP infusion alone.

following these manipulations (Table 1). These apparent rises in GFR did not differ significantly from time-matched measurements in the vehicle-treated group, and probably represent a 'wash-out' effect from an increase in urine flow.

The small fall in blood pressure produced by ANP $100 \text{ ng kg}^{-1} \text{ min}^{-1}$ was not significantly changed by the addition of candoxatrilat or SC 46542. The combination of candoxatrilat and SC 46542 had no significant hypotensive effect (Table 1).

Candoxatrilat and SC 46542 in the A-V fistula rat

Typical of this model of heart failure, the A-V fistula rats exhibited cardiac hypertrophy compared to the sham-operated animals (mean heart weight fistulas vs shams; 1.46 ± 0.07 vs $1.08 \pm 0.02 \text{ g}$, $P < 0.01$). Plasma ANP concentration in the A-V fistula rats sampled was $833 \pm 105 \text{ pg ml}^{-1}$ compared with $118 \pm 33 \text{ pg ml}^{-1}$ in the sham-operated animals. Consistent with raised circulating levels of ANP, the A-V fistula rats also had a higher 24 h urinary cyclic GMP excretion (181 ± 23 vs $35 \pm 8 \text{ nmol}$, $P < 0.01$). Twenty four h urinary cyclic GMP excretion and heart weights did not differ significantly between the A-V fistula treatment groups.

Urinary sodium excretion in the A-V fistula rats receiving saline alone was relatively stable (Figure 5). In contrast to the sham-operated animal, bolus administration of candoxatrilat (3 mg kg^{-1}) to the A-V fistula rat produced a prompt and sustained rise in urinary sodium excretion, accompanied

Figure 4 Effect of candoxatrilat (3 mg kg^{-1} , $\circ$) and SC 46542 ($680 \mu\text{g kg}^{-1}$, then $68 \mu\text{g kg}^{-1} \text{ min}^{-1}$, $\blacksquare$) alone and in combination ($\square$) on urinary sodium (Na^+), cyclic GMP and fractional lithium (Li^+) excretion in conscious normal rats. Data are mean with s.e.mean shown by vertical bars. $*P < 0.05$, $**P < 0.01$ compared to candoxatrilat or SC 46542 alone.

by a further rise in urinary cyclic GMP excretion (Figure 5).

Similarly, SC 46542 also stimulated a natriuresis and rise in urinary cyclic GMP excretion in the A-V fistula animal but compared with candoxatrilat, there appeared to be some loss of potency with a shift in the dose-response curve to the right (Figure 6), in that the lower dose of SC 46542, which was equipotent with candoxatrilat 3 mg kg^{-1} in the normal rat co-infused with low-dose ANP, had no significant effect on sodium excretion in the A-V fistula model. Increasing the dose 10 fold, however, produced a response closer to that seen with the E-24.11 inhibitor. The natriuretic response to the combination of candoxatrilat and high-dose SC 46542 in the A-V fistula rat was not significantly greater than that produced by either compound alone. Thus, the peak mean sodium excretion with the combined treatment was $12.2 \pm 1.8 \mu\text{mol min}^{-1}$ compared to $10.2 \pm 1.5 \mu\text{mol min}^{-1}$ with candoxatrilat 3 mg kg^{-1} given alone.

Baseline mean arterial blood pressure in the A-V fistula rats receiving vehicle was $103 \pm 4 \text{ mmHg}$, and 103 ± 5 , 102 ± 4 and $105 \pm 5 \text{ mmHg}$ in the candoxatrilat and low and high dose SC 46542 treatment groups respectively. This compares with $112 \pm 4 \text{ mmHg}$ in the sham-operated animals (NS). Blood pressure in the A-V fistula rats receiving active treatment did not differ significantly at any time from the fistula control (vehicle alone) group.

Baseline GFR was $2.8 \pm 0.3 \text{ ml min}^{-1}$ in the A-V fistula rats receiving saline and 3.0 ± 0.3 , 2.8 ± 0.4 and $2.8 \pm 0.3 \text{ ml min}^{-1}$ in the candoxatrilat and low and high dose SC 46542 groups respectively, compared to $3.2 \pm 0.3 \text{ ml min}^{-1}$ in the

Figure 5 Effect of candoxatrilat (3 mg kg^{-1}) on urinary sodium (Na^+) and cyclic GMP excretion in A-V fistula ($\square$) and sham-operated rats ($\bullet$). Control A-V fistula rats received saline alone throughout ($\circ$). Data are mean with s.e.mean shown by vertical bars. $*P < 0.05$, $**P < 0.01$ compared to A-V fistula controls.

Figure 6 Effect of two doses of SC 46542 ($68 \mu\text{g kg}^{-1}$ bolus then $6.8 \mu\text{g kg}^{-1} \text{ min}^{-1}$, $\triangle$; or $680 \mu\text{g kg}^{-1}$ then $68 \mu\text{g kg}^{-1} \text{ min}^{-1}$, $\square$) on urinary sodium (Na^+) and cyclic GMP excretion in A-V fistula rats. Sham-operated rats received the higher dose of SC 46542 alone ($\bullet$). Control A-V fistula rats received vehicle alone throughout ($\circ$). Data are mean with s.e.mean shown by vertical bars. $*P < 0.05$, $**P < 0.01$ compared to A-V fistula controls.

sham-operated rats. None of the treatments significantly altered GFR when compared with the saline controls.

Discussion

Candoxatrilat and SC 46542 inhibit two major mechanisms for clearing ANP – the enzyme E-24.11 and C-ANP receptors respectively. Given alone in the presence of 'physiological' plasma ANP levels, neither candoxatrilat nor SC 46542 significantly affected sodium excretion. It has been suggested that under these conditions, C-ANP receptors are capable of buffering the effects of E-24.11 inhibition on plasma ANP clearance and *vice versa*. In support of this, combined inhibition of both clearance pathways has been reported to produce a greater rise in plasma ANP (Koepke *et al.*, 1989; Seymour *et al.*, 1991) and, as shown in this study, a greater natriuretic response than inhibition of either pathway alone. Similarly, when plasma ANP levels are elevated, either by infusion of exogenous peptide or in pathological states such as heart failure, the capacity of both clearance mechanisms becomes more saturated; the ability of one to compensate for inhibition of the other is limited, and the natriuretic potential of candoxatrilat and SC 46542 is unmasked.

In normal rats with plasma ANP levels elevated 5 fold by infusion of exogenous peptide, SC 46542 showed a trend towards producing a greater natriuresis than candoxatrilat, although this did not reach statistical significance. A greater natriuretic response to SC 46542 might be anticipated from data on the effects of E-24.11 inhibition and C-ANP receptor ligands on the pharmacokinetics of ANP. While both E-24.11 inhibition and the C-ANP receptor ligand, C-ANP(4-23), reduce the clearance of ANP, C-ANP(4-23) in addition reduces the volume of distribution of the peptide leading to a greater increase in plasma ANP levels (Chiu *et al.*, 1991).

However, the present studies suggest that neither candoxatrilat nor SC 46542 act simply through increasing plasma ANP levels. Although the addition of candoxatrilat or SC 46542 to ANP 100 ng kg⁻¹ min⁻¹, and the combination of candoxatrilat and SC 46542, enhanced urinary sodium and cyclic GMP excretion to levels similar to, or greater than, those achieved with ANP 300 ng kg⁻¹ min⁻¹, these compounds did not reproduce all the effects of the high-dose ANP infusion. First, the increased renal response was produced without matching the fall in blood pressure seen with the higher dose of ANP. The effect of infused ANP on blood pressure is due mainly to a reduction in cardiac output mediated by the action of the circulating peptide on vascular resistance and capillary absorption (Chien *et al.*, 1987; Tripodo & Barbee, 1987). E-24.11 inhibitors (SQ 29072 and SCH 34826) have been reported to increase and prolong the hypotensive response to bolus administration of high doses of ANP in the spontaneously hypertensive rat (SHR) (Seymour *et al.*, 1990) and add to the hypotension produced by C-ANP(4-23) in DOCA-salt hypertensive rats (Seymour *et al.*, 1991; Subbarao *et al.*, 1991). In contrast, other authors have found that co-administration of thiorphan with a low-dose ANP infusion or SC 46542 does not significantly affect blood pressure in normotensive rats (Trapani *et al.*, 1989; Koepke *et al.*, 1989). The explanation for this difference in response between normotensive and hypertensive rats is not known, although it has been suggested that DOCA-salt rats are particularly sensitive to the vascular effects of ANP (Kaneko *et al.*, 1987; Seymour *et al.*, 1991). Nonetheless, the consensus from these data is that in conscious normotensive rats, compared with pharmacological doses of ANP, candoxatrilat and SC 46542 preferentially enhance the renal effects of ANP.

Second, comparison of the renal effects of candoxatrilat or SC 46542 with low-dose ANP and the higher dose ANP infusion suggest that they differ in the renal mechanism by which sodium excretion is increased; that is, each may facilitate a different action of ANP within the kidney. Thus

the natriuretic response to the higher dose of ANP was accompanied by a significant rise in GFR but no significant change in FeLi when compared to vehicle-treatment alone. Candoxatrilat and SC 46542 increased sodium excretion without any significant change in GFR and candoxatrilat markedly increased FeLi when given with ANP. The natriuretic response to ANP is thought to result from actions on both the glomerulus and tubule (Cogan, 1990). A rise in GFR is a consistent finding with high doses of ANP (Cogan, 1990) and may be the principal reason for the increased natriuretic response to increasing doses of the peptide. The favoured tubule site of action of ANP is the inner medullary collecting duct, as functional receptors for the peptide have been found there (Zeidel, 1990). However, ANP has also been reported to increase FeLi (Harris *et al.*, 1988), evidence of a pre-distal tubule site of action. The use of FeLi to define the tubule site of action of a drug is not without controversy, not least because doses of lithium that are natriuretic have been reported to inhibit the natriuretic effects of ANP in man (Freestone *et al.*, 1990). The doses of lithium infused in our studies resulted in plasma lithium concentrations <0.2 mmol l⁻¹, i.e. below levels that have been shown to affect sodium excretion (Freestone *et al.*, 1990), and the response to ANP is comparable to our experience in other experiments where lithium was not included in the infusate (Wilkins *et al.*, 1990b). An increase in FeLi with ANP treatment has not been observed by all authors (Cavero *et al.*, 1990) and no clear effect on FeLi was apparent in the studies reported here. This inconsistency may be due to the opposing effects of volume depletion, which increases lithium reabsorption in the distal tubule (Thomsen, 1990), thus reducing overall lithium clearance; no attempt was made to replace volume loss in the present experiments and this may have reduced the effect of ANP on FeLi. Nonetheless, in the same protocol, a marked rise in FeLi was seen with candoxatrilat when given with ANP, suggesting a pronounced pre-distal tubule effect with this drug. A similar rise in FeLi has been observed in a dog model of heart failure with the E-24.11 inhibitor, SQ 28,603 (Cavero *et al.*, 1990). The most likely site of this effect is the proximal tubule. First, E-24.11 is found in great abundance in the renal brush border. Its physiological role at this site is unknown but it is ideally placed to modify the effects of peptides on proximal tubule function. Second, ANP has been shown to antagonize the effect of angiotensin II on sodium reabsorption in the proximal tubule (Garvin, 1989).

The effect of candoxatrilat on FeLi was not shared by SC 46542. The mechanism of the increased natriuretic effect of this ligand is not apparent from this study. It has been proposed that a component of the renal response to ANP may arise from an action on the vasa recta, causing an increase in peritubular capillary hydrostatic pressure and/or an increase medullary blood flow, both of which would favour net sodium excretion (Zeidel, 1990). Given the preponderance of C-ANP receptors in the glomerulus (Rutherford *et al.*, 1991), it is tempting to suggest that inhibition of ANP clearance by these receptors may allow increased delivery of the peptide to the vasa recta, so enhancing these effects, although this remains speculative. An interesting observation is that the combination of candoxatrilat and SC 46542 markedly increased sodium excretion without affecting FeLi, suggesting that the major contribution to the natriuretic effect of this combination came from the C-ANP receptor ligand. In this context it is important to note that SC 46542, like ANP, is a substrate for E-24.11 (A.J. Kenny, personal communication) and would be protected from metabolism by candoxatrilat. Thus, as well as a response to combined inhibition of both clearance pathways, part of the increased response to co-administration of SC 46542 and candoxatrilat may arise from higher plasma levels of SC 46542.

Further evidence that candoxatrilat, SC 46542 and infusions of pharmacological doses of ANP differ in their action

on the kidney comes from the response to these agents in the A-V fistula rat. Consistent with the clinical condition, it has been shown that this model of heart failure is hyporesponsive to infusions of ANP (Wilkins *et al.*, 1990b). In this earlier study, increasing doses of ANP (100 to 1000 ng kg⁻¹ min⁻¹) produced a 2 fold increase in sodium excretion in the A-V fistula animal compared to a 4 fold rise in the sham-operated rat. The mechanisms underlying the resistance to this peptide in heart failure are thought to include renal perfusion pressure, receptor down-regulation, increased activity of the renin-aldosterone system and increased renal sympathetic nerve activity. On the other hand, both candoxatrilat and SC 46542 were effective in stimulating a natriuretic response in the A-V fistula rat. Compared to candoxatrilat, there was some resistance to the natriuretic effect of the C-ANP receptor ligand: SC 46542, in a dose that was equipotent with candoxatrilat in producing a natriuresis in the normal rat receiving exogenous ANP, produced only a small, non-significant rise in sodium excretion in the A-V fistula rat. However, increasing the dose 10 fold induced a natriuresis that matched that produced by the E-24.11 inhibitor. The possibility that these compounds enhance other natriuretic factors, in addition to ANP, in A-V fistula animals cannot be excluded but the accompanying rise in urinary cyclic GMP is commensurate with an ANP-mediated response. Interest-

ingly, co-treatment with the two compounds had a less than additive effect in this model. While this would indicate that their renal effects are not completely independent of each other, for example, a component of the response to both compounds is mediated by a rise in plasma ANP levels, a confounding factor in these experiments is the accompanying volume depletion which acts to limit the natriuresis achieved.

In summary, inhibition of E-24.11 with candoxatrilat or C-ANP receptors with SC 46542 enhanced the natriuretic but not the hypotensive effects of low-dose ANP infusion in the rat and induced a natriuresis in the rat A-V fistula model of heart failure. These effects contrast with the response to infusions of pharmacological doses of the peptide and indicate that E-24.11 inhibitors and C-ANP receptor ligands have actions other than those produced by increasing circulating ANP levels. The marked increase in FeLi with candoxatrilat plus ANP and lack of effect of this combination on GFR suggest that candoxatrilat preferentially increases a tubule action of ANP. The mechanism by which SC 46542 increase the natriuretic response to ANP remains to be elucidated.

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