Clearance Mechanisms of Atrial and Brain Natriuretic Peptides in Rats

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To assess clearance mechanisms of atrial and brain natriuretic peptides in the circulation, we examined the effects of a neutral endopeptidase (NEP) inhibitor and a clearance receptor ligand on plasma concentrations of the peptides in normal rats. Plasma concentrations of endogenous α-rat atrial natriuretic peptide (α-rANP) were not significantly elevated by intravenous infusion of a NEP inhibitor, phosphoramidon, but were elevated threefold by intravenous infusion of a clearance receptor ligand, des(Gln^{18}-Gly^{22})-rANP$_{(4-23)}$-NH$_2$ [C-ANF$_{(4-23)}$]. On the other hand, the clearance of α-rANP given intravenously at the pharmacological dose, 600 pmol/min/kg for 2 min, was decreased to one-third by the administration of phosphoramidon, although the administration of C-ANF$_{(4-23)}$ did not significantly decrease the clearance. The clearance of rat brain natriuretic peptide (rBNP) given at 600 pmol/min/kg for 2 min was approximately 38% lower than that of α-rANP. The effect of phosphoramidon on the clearance of rBNP was not significant and was similar to that of C-ANF$_{(4-23)}$. These results suggest that clearance receptor is involved in the clearance of the physiological levels of α-rANP and that NEP plays a major role in the clearance of a pharmacological dose of α-rANP, at which clearance receptors are thought to be saturated, and also indicate a pharmacokinetic difference between α-rANP and rBNP.

KEY WORDS: atrial natriuretic peptide; brain natriuretic peptide; clearance receptor; neutral endopeptidase; phosphoramidon.

INTRODUCTION

The vasodilating and diuretic actions of atrial natriuretic peptide (ANP)[4] are therapeutic for some pathological conditions including congestive heart failure (1). However, clinical application of ANP is limited because of its short biological half-life (2). The rapid inactivation of this circulating peptide is attributed to receptor-mediated internalization and enzymatic degradation. ANP binds not only to biologically active receptors associated with guanylate cyclase activity but also to biologically silent clearance receptors (3,4). Maack *et al.* (3) demonstrated that a ring-deleted and C terminal-truncated ANP analogue, des(Gln^{18}-Gly^{22})-rANP$_{(4-23)}$-NH$_2$ [C-ANF$_{(4-23)}$], binds with a high affinity in the isolated perfused rat kidney but does not have renal effects or antagonize the natriuretic action of ANP in the same preparation. They also found that intravenous infusion of C-ANF$_{(4-23)}$ to anesthetized rats results in natriuresis due to a rise in endogenous ANP. On the other hand, the enzymatic degradation is attributed mainly to neutral endopeptidase EC 3.4.24.11 (NEP), which is known to inactivate the 28-amino acid α-human ANP (α-hANP) and α-rat ANP (α-rANP) (5). Vanneste *et al.* (6) reported that purified NEP hydrolyzes several sites, including the Cys^7–Phe^8 bond in the ring structure, which is indispensable for the biological action of α-ANP. In the present study, using the specific clearance receptor ligand, C-ANF$_{(4-23)}$, and the NEP inhibitor, phosphoramidon, we examined the contribution of the two clearance mechanisms on the pharmacokinetics of α-rANP as well as rat brain natriuretic peptide (rBNP), which has a lower clearance than α-rANP (7).

MATERIALS AND METHODS

Animals

Male Wistar rats, each weighing 300–350 g, were used. Prior to the experiments, the rats were housed in a temperature- and humidity-controlled room with free access to water and standard rat chow.

Materials

Sodium pentobarbital was purchased from Abbott (Chicago, IL). Phosphoramidon, carbobenzoxy-Gly-Gly-Leu-*p*-nitroanilide, α-rANP, and 45-amino acid rBNP were purchased from Peptide Institute (Osaka, Japan). Bovine serum albumin was obtained from Sigma (St. Louis, MO). Aminopeptidase M was purchased from Boehringer Mannheim (Germany). All other chemicals were the highest purity available.

Inhibition of NEP by Phosphoramidon

Phosphoramidon was dissolved in isotonic saline and infused via a femoral vein at a constant rate of 110 μL/min to inhibit the metabolism of natriuretic peptides (8). The administration protocol consisted of a loading dose of 825 nmol/min/kg for 2 min, followed by a maintenance dose of 165 nmol/min/kg throughout the experiment, including a 20-min stabilizing period before the administration of natriuretic peptides, except where specified otherwise.

Blocking of Clearance Receptor-Mediated Internalization by C-ANF$_{(4-23)}$

C-ANF$_{(4-23)}$ was intravenously infused to block clearance of receptor-mediated internalization of natriuretic peptides (4). A loading dose of C-ANF$_{(4-23)}$ of 15.7 nmol/min/kg for 2 min was followed by a maintenance dose of 3.13 nmol/min/kg.

Experimental Design

Pharmacokinetics of α-rANP and rBNP were investi-

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[4] Abbreviations used: ANP, atrial natriuretic peptide; α-rANP, α-rat ANP; α-hANP, α-human ANP; BBM, brush border membranes; BNP, brain natriuretic peptide; rBNP, rat BNP; C-ANF$_{(4-23)}$, des-(Gln^{18}-Gly^{22})-rANP$_{(4-23)}$-NH$_2$; f_u, fraction unbound; NEP, neutral endopeptidase.

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gated in rats anesthetized with 60 mg/kg sodium pentobarbital. The natriuretic peptide was diluted with phosphate-buffered saline (pH 7.4) containing 0.1% bovine serum albumin, and 600 pmol/min/kg was administered for 2 min via a jugular vein. This dose was selected so that the administered peptide did not cause severe hypotension and that the plasma α-rANP and rBNP were measurable during the experimental period (7). Blood samples (0.4 mL) were withdrawn via a catheter in a femoral artery just before administration of natriuretic peptides and 1, 2, 3, 4, 5, 7, 10, and 15 min after the start of the infusion.

To examine the extent of contribution of NEP and clearance receptors to the clearance of natriuretic peptides, we tested the effects of phosphoramidon and C-ANF$_{(4-23)}$ on the baseline (endogenous) natriuretic peptide concentration and on the pharmacokinetics of exogenous natriuretic peptides. We used four protocols: (i) no treatment (control), (ii) administration of phosphoramidon (phosphoramidon), (iii) administration of C-ANF$_{(4-23)}$ (C-ANF$_{(4-23)}$), and (iv) coadministration of C-ANF$_{(4-23)}$ and phosphoramidon (combination).

Plasma Protein Binding of Phosphoramidon

Plasma protein binding of phosphoramidon was measured by the ultrafiltration technique using a commercial kit (Ultrafree C3LGC, Millipore, Bedford, MA). A small volume of phosphoramidon solution was added to plasma, and a 1-mL sample placed in the device was centrifuged at room temperature. About 100 μL of the ultrafiltrate was obtained for the determination of the plasma unbound phosphoramidon concentration. The adsorption of phosphoramidon onto the ultrafiltration membrane was negligible.

Assay of Phosphoramidon

The inhibitory activity of phosphoramidon for rat NEP was assessed using renal brush border membranes (BBM) as the enzyme source. The two-step enzyme assay was based on the photogenic substrate, carbobenzoxy-Gly-Gly-Leu-*p*-nitroanilide. BBM were isolated from the rat renal cortex as described previously (9) and were suspended in a buffer consisting of 50 m*M* Tris (pH 7.4) and 2.5 m*M* $CaCl_2$. A 400-μL aliquot of BBM suspension containing approximately 300 μg of protein was incubated with the substrate (50 μL of 2 m*M* solution dissolved in dimethylformamide) and 50 μL of the sample or the solution of phosphoramidon (0.01–10 μ*M*) for 60 min at 37°C. The reaction was terminated by the addition of 25 μL of a solution of phosphoramidon (2.2 m*M*), and Leu-*p*-nitroanilide released during the first step was hydrolyzed by the addition of 25 μL of a solution of aminopeptidase M (100 μg/mL) for a further 30 min at 37°C. After the addition of acetonitrile (550 μL), the released *p*-nitroaniline was assayed at an absorbance wavelength of 410 nm.

Assay of α-rANP and rBNP

Plasma concentrations of α-rANP and rBNP were determined by radioimmunoassay as described previously (2,10).

Data Analysis

The plasma concentration of phosphoramidon after infusion at a constant rate was fitted to the following equation:

$$C = I/\mathrm{CL} \cdot (1 - e^{-\mathrm{CL}/V \cdot t}) \quad (1)$$

where C and I are the plasma concentration and the infusion rate of phosphoramidon, respectively, and t is the duration of the infusion. V and CL are the volume of distribution and the total clearance, respectively.

The disposition of α-rANP and rBNP following intravenous infusion was fitted to the following equation as described previously (2,7):

$$C = C_1 \cdot (e^{-\lambda_1 \cdot t'} - e^{-\lambda_1 \cdot t}) + C_2 \cdot (e^{-\lambda_2 \cdot t'} - e^{-\lambda_2 \cdot t}) + C_3 \quad (2)$$

where C is the plasma concentration of natriuretic peptides, t is the time from the start of infusion, t' is 0 for t equal to and less than the infusion time (T), and t' is $t - T$ for t larger than T. C_1 and λ_1 are the early phase variables, and C_2 and λ_2 are the later phase variables. C_3 is the baseline concentration before administration of α-rANP. C_3 was omitted for the pharmacokinetic analysis of rBNP since the baseline concentrations were below the detection limit. The predicted steady-state concentration of the exogenous natriuretic peptide following the infusion with infinite T is $C_1 + C_2$. Therefore, the total clearance (CL) of natriuretic peptides was calculated as follows:

$$\mathrm{CL} = I/(C_1 + C_2) \quad (3)$$

where I is the infusion rate of natriuretic peptides.

The nonlinear least-squares regression was analyzed using Nelder–Mead's algorithm (11) on a FACOM M780 computer (Kyoto University Data Processing Center). Values are expressed as mean ± SE. Significance of differences between mean values was calculated by a nonpaired t test. Multiple comparison was performed using a Scheffé-type test. A P value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

In our previous study, we demonstrated that the disappearance of rBNP after intravenous administration was slower than that of α-rANP and that its clearance was lower than that of α-rANP (7). In the present study, using the specific clearance receptor ligand, C-ANF$_{(4-23)}$, and the NEP inhibitor, phosphoramidon, we compared the respective clearance mechanisms of α-rANP and rBNP.

Phosphoramidon is a potent and specific NEP inhibitor (5,8). Figure 1 shows the standard curve for NEP activities vs phosphoramidon concentrations from three separate experiments. Phosphoramidon completely inhibited the hydrolysis of carbobenzoxy-Gly-Gly-Leu-*p*-nitroanilide (200 μ*M*), with an IC_{50} (the concentration showing 50% inhibition of control value) of 54.4 ± 7.9 n*M* (n = 3) for the NEP of rat renal BBM. In addition, no inhibition of NEP activity by C-ANF$_{(4-23)}$ was observed in the concentration range used (Fig. 1).

First, we studied the pharmacokinetics of phosphoramidon in rats following constant intravenous infusion, 165 nmol/min/kg (Fig. 2). Equation (1) was used for the pharma-

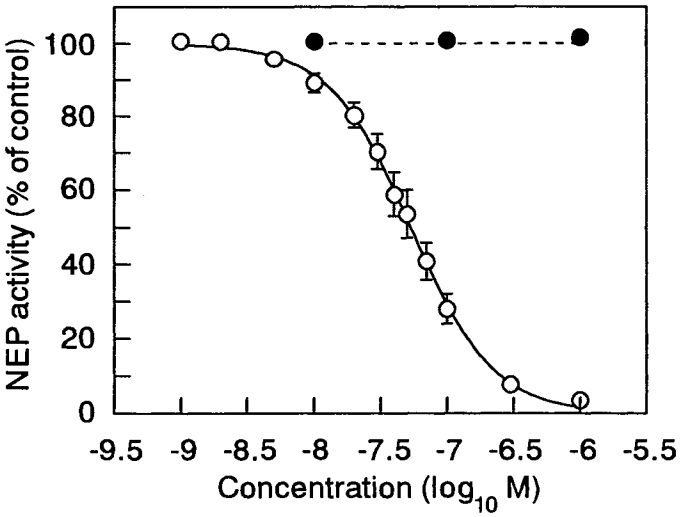

Fig. 1. Standard curve for neutral endopeptidase activity vs phosphoramidon (open circles) or C-ANF$_{(4-23)}$ (filled circles) concentration. Values are mean of three experiments. Vertical bars represent SE.

cokinetic analysis, and the estimated V and CL of phosphoramidon was 229 ± 36 mL/kg (n = 5) and 19.3 ± 1.6 mL/min/kg (n = 5), respectively. The unbound fraction (f_u) of phosphoramidon in plasma was not dependent on its concentration at a range from 2 to 10 μM, and the mean f_u was 0.280 ± 0.017 (n = 15). Figure 2 also shows the simulated unbound plasma concentration of phosphoramidon following intravenous infusion (165 nmol/min/kg), which was calculated using V, CL, and f_u. The predicted mean concentration

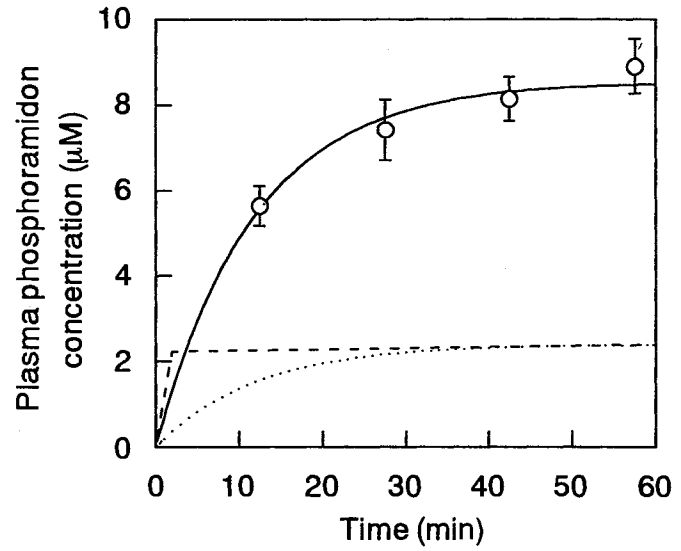

Fig. 2. Time course of mean (±SE) plasma phosphoramidon concentration following intravenous infusion given at 165 nmol/min/kg in rats (n = 5). The solid line is the simulated plasma phosphoramidon concentration based on Eq. (1). The dotted line is the simulated plasma unbound phosphoramidon concentration following constant intravenous infusion at 165 nmol/min/kg, and the dashed line is the simulated plasma unbound phosphoramidon concentration following a loading dose of 825 nmol/min/kg for 2 min, followed by a maintenance dose of 165 nmol/min/kg.

of unbound phosphoramidon at steady state was 2.4 μM and 44-fold higher than the IC_{50} value for rat NEP, suggesting the complete inhibition of NEP activity. For the rapid inhibition of NEP by phosphoramidon, an administration protocol consisting of a loading dose of 825 nmol/min/kg for 2 min, followed by a maintenance dose of 165 nmol/min/kg, was used in the subsequent experiments. The dashed line shown in Fig. 2 is the simulated concentration of unbound phosphoramidon with this administration protocol.

C-ANF$_{(4-23)}$ is a specific ligand of clearance receptors with a K_i value of 100 pM for the binding of α-rANP to rat clearance receptors (solubilized membranes from rat aortic smooth muscle cell) (4). Maack *et al.* (3) showed that intravenous infusion of C-ANF$_{(4-23)}$ at a rate of 1 μg/min/kg (0.627 nmol/min/kg) in rats blocks the clearance receptors. In the present study, we used an administration protocol consisting of a loading dose at 15.7 nmol/min/kg for 2 min, followed by a maintenance dose at 3.13 nmol/min/kg, to accomplish rapid and complete inhibition of clearance receptors.

Table I shows the effect of phosphoramidon, C-ANF$_{(4-23)}$, and their coadministration on the endogenous (baseline) plasma α-rANP concentration before its administration. Treatment with phosphoramidon alone did not significantly increase the plasma α-rANP concentration, whereas treatment with C-ANF$_{(4-23)}$ resulted in a threefold increase. The plasma α-rANP concentration after coadministration of phosphoramidon and C-ANF$_{(4-23)}$ was only slightly higher than that in the C-ANF$_{(4-23)}$-treated rat. These results suggest that clearance receptors are largely involved in the clearance of endogenous α-rANP.

Suga *et al.* (4) reported that α-rANP binds with a high affinity to rat, bovine, and human clearance receptors and that the K_d value for binding to rat clearance receptors is 6.6 pM. The endogenous α-rANP concentration observed in the present study (20.4 pM) was only threefold higher than the K_d value. The effect of C-ANF$_{(4-23)}$ treatment on the endogenous α-rANP concentration was comparable with that reported by Maack *et al.* (3). In addition, Chiu *et al.* (12) reported that the clearance of a tracer dose of ^{125}I-α-rANP was significantly decreased by the administration of C-ANF$_{(4-23)}$ in rats but only slightly decreased by the administration of another NEP inhibitor, SCH39370. These findings suggest that clearance receptors are not saturated and avidly clear α-rANP in the lower concentration range or at physiological levels.

Figure 3 shows the effect of phosphoramidon, C-ANF$_{(4-23)}$, and their combination on the time course of the plasma α-rANP concentration following the intravenous

Table I. Effect of Phosphoramidon and C-ANF$_{(4-23)}$ on Endogenous Plasma α-rANP Concentrations in Rats[a]

	α-rANP concentration (pM)
Control (n = 7)	20.4 ± 1.9
Phosphoramidon (n = 6)	30.3 ± 3.0
C-ANF$_{(4-23)}$ (n = 5)	64.5 ± 8.8*
Combination (n = 5)	86.0 ± 11.1*

[a] Results are presented as mean ± SE.
[b] Significantly different from the control group, $P < 0.05$.

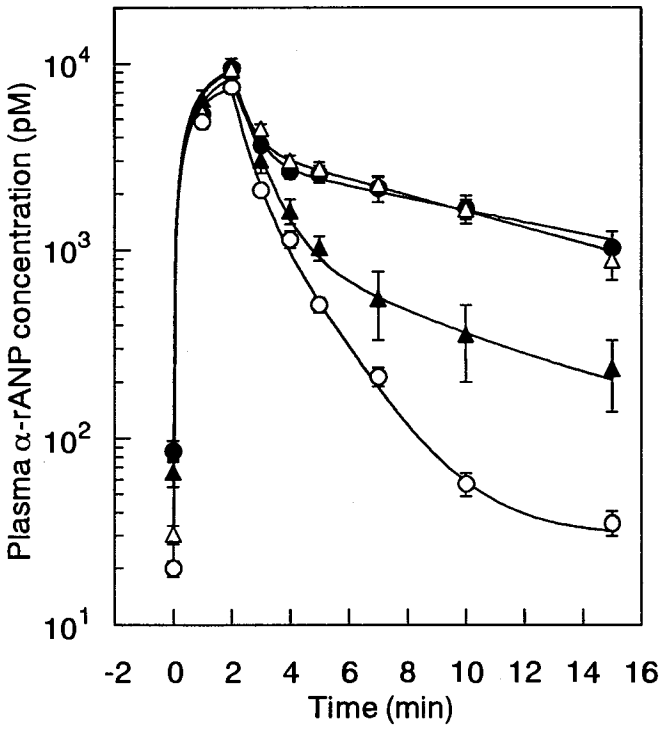

Fig. 3. Time course of mean (±SE) plasma concentration of α-rANP following intravenous infusion of the peptide (600 pmol/min/kg from time 0 to time 2) in the presence of phosphoramidon and C-ANF$_{(4-23)}$. Control (open circles), phosphoramidon (open triangles), C-ANF$_{(4-23)}$ (filled triangles), and the combination (filled circles). Values are means of five to seven rats.

infusion of exogenous α-rANP (600 pmol/min/kg for 2 min). Table II shows the clearance values in this experiment. The administration of phosphoramidon considerably prolonged the half-time of plasma disappearance of α-rANP in the later (λ_2) phase: 6.74 ± 0.93 min (n = 6) in the phosphoramidon-treated group vs 1.35 ± 0.09 min (n = 7) in the control group. The plasma clearance of α-rANP was decreased to one-third by the administration of phosphoramidon. On the other hand, the effect of C-ANF$_{(4-23)}$ on the disappearance and the plasma clearance of α-rANP was less than that of phosphoramidon. The time course of the plasma α-rANP concentration and the clearance after coadministration of phosphoramidon and C-ANF$_{(4-23)}$ were similar to those after administration of phosphoramidon alone. These findings suggested that NEP plays a major role in the clearance of α-rANP in the higher plasma concentration range or at pharmacological levels, at which clearance receptors are thought to be saturated.

Table II. Effect of Phosphoramidon and C-ANF$_{(4-23)}$ on Clearance of α-rANP and rBNP Following Intravenous Infusion of Peptide (600 pmol/min/kg for 2 min) in Rats[a,*]

	Clearance (mL/min/kg)	
	α-rANP	rBNP
Control	74.9 ± 4.9 (7)	45.9 ± 1.9 (4)[b]
Phosphoramidon	26.3 ± 2.4 (6)[a]	31.2 ± 3.2 (5)
C-ANF$_{(4-23)}$	53.4 ± 5.5 (5)	32.8 ± 4.9 (4)[b]
Combination	26.1 ± 5.1 (5)[a]	23.3 ± 2.6 (4)[a]

[a] The number of animals is given in parentheses, and values are expressed as mean ± SE.

* Superscript a, significantly different from the control group, $P < 0.05$; b, significantly different from the value for the α-rANP-treated group, $P < 0.05$.

The 45-amino acid rBNP is the predominant circulating molecular form of rBNP (13,14). However, in the present study, we could not detect endogenous rBNP before its administration. Yokota *et al.* (14) reported that it was impossible to obtain enough plasma to determine the endogenous rBNP levels in individual normal rats and that the endogenous plasma rBNP concentration (0.73 p*M*) determined with a plasma sample from several rats was much lower than the α-rANP concentration (29.8 p*M*). In the present study, therefore, the pharmacokinetics of rBNP was investigated by exogenous administration of the peptide.

Figure 4 shows the effect of phosphoramidon, C-ANF$_{(4-23)}$, and their combination on the time course of plasma rBNP concentration following intravenous infusion of exogenous rBNP (600 pmol/min/kg for 2 min). Table II shows the clearance values in this experiment. The administered rBNP was cleared more slowly than α-rANP, and the half-time of plasma disappearance in the later (λ_2) phase was fourfold longer for rBNP than for α-rANP: 5.45 vs 1.35 min. In addition, the clearance value of rBNP was approximately 38% lower than that of α-rANP. The effect of NEP inhibition on the pharmacokinetics of rBNP was lesser than that on the

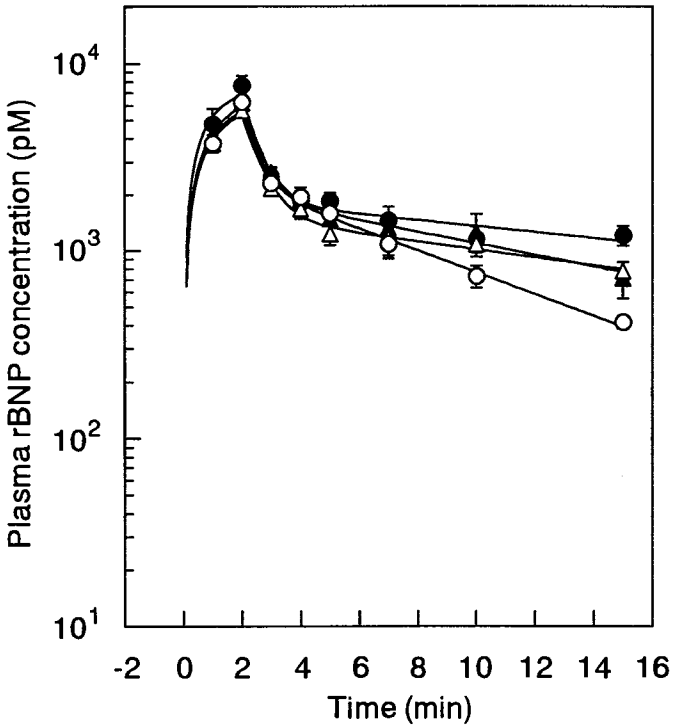

Fig. 4. Time course of mean (±SE) plasma concentration of rBNP following intravenous infusion of the peptide (600 pmol/min/kg from time 0 to time 2) in the presence of phosphoramidon and C-ANF$_{(4-23)}$. Control (open circles), phosphoramidon (open triangles), C-ANF$_{(4-23)}$ (filled triangles), and the combination (filled circles). Values are means of four or five rats.

pharmacokinetics of α-rANP. The administration of phosphoramidon minimally prolonged the plasma disappearance of rBNP, and only slightly decreased its clearance. In addition, the clearance of rBNP after administration of phosphoramidon was not significantly different from that of α-rANP: 31.2 mL/min/kg (rBNP) vs 26.3 mL/min/kg (α-rANP). The effect of C-ANF$_{(4-23)}$ on the pharmacokinetics of rBNP was similar to that of phosphoramidon; however, the clearance of rBNP in C-ANF$_{(4-23)}$-treated rats was significantly different from that of α-rANP: 32.8 mL/min/kg (rBNP) vs 53.4 mL/min/kg (α-rANP). These findings suggest that the pharmacokinetic difference between α-rANP and rBNP is due mainly to a more efficient NEP-mediated mechanism for the degradation of α-rANP compared with that of rBNP. Since the degradation of natriuretic peptides by NEP is highly dependent on the structures of natriuretic peptides (15,16), further understanding of the pharmacokinetic difference of natriuretic peptides awaits studies on the affinity of BNP to NEP in addition to clearance receptors (4).

In conclusion, the present study demonstrates different major mechanisms involved in the pharmacokinetics of α-rANP and rBNP, which may help us understand pharmacological and pathophysiological functions of these natriuretic peptides on the pharmacokinetic basis.

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