# Kinins mediate the inhibition of atrial natriuretic peptide diuretic effect induced by pepsanurin

MAURICIO P BORIC[1], HECTOR R CROXATTO, JOSE M MORENO, ROSA SILVA, CRISTIAN HERNANDEZ and JUAN S ROBLERO

Unidad de Regulación Neurohumoral, Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

*Pepsanurin is a peptidic fraction resulting from pepsin digestion of plasma globulins, that inhibits ANP renal excretory actions. We studied whether kinin-like peptides mediate the anti-ANP effect by testing if pepsanurin: 1) was blocked by the kinin B2 receptor antagonist HOE-140, 2) was produced from kininogen, and 3) was mimicked by bradykinin. Anti-ANP activity was assessed in anesthetized female rats by comparing the excretory response to two ANP boluses (0.5 μg iv) given before and after ip injection of test samples. Pepsanurin from human or rat plasma (1-5 mL/kg), and bradykinin (5-20 μg/kg), dose-relatedly inhibited ANP-induced water, sodium, potassium and cyclic GMP urinary excretion, without affecting arterial blood pressure. The same effect was exerted by pepsin hydrolysates of purified kininogen, whereas hydrolysates of kininogen-free plasma had no effect. HOE-140 (5 μg, iv) did not alter baseline, or ANP-induced excretion, but blocked the anti-ANP effects of pepsanurin. Histamine (15 μg/kg) plus seroalbumin hydrolysates did not affect ANP response, despite inducing larger peritoneal fluid accumulation as compared with pepsanurin or bradykinin. We concluded that kinins cleaved from kininogen mediate the anti-ANP effects of pepsanurin by activation of kinin B2 receptors, independently of changes in systemic arterial pressure or peritoneal fluid sequestration.*

***Key terms:*** *atriopeptin, bradykinin, HOE-140, kinin B2 receptor antagonist, kininogen, natriuresis, pepsanurin*

## INTRODUCTION

Pepsanurin is a peptidic fraction produced by pepsin hydrolysis of blood plasma, or plasma globulins, that delays the excretion of a water load when injected ip in conscious hyperhydrated rats (8). Pepsanurin antagonizes the excretory effect of atrial natriuretic peptide (ANP) when injected ip (6) or intraduodenally (id) (9) in anesthetized rats.

Pepsanurin does not affect arterial pressure nor baseline renal function, but abolishes the increase in glomerular filtration rate and blunts the rise in fractional sodium excretion induced by ANP (6). In addition, pepsanurin blocks ANP actions in isolated perfused rat kidneys (6), suggesting an intrarenal mechanism of action related and opposed to ANP actions at the glomerulus and distal nephron.

[1] **Correspondence to:** Dr Mauricio P Boric, Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas, P Universidad Católica de Chile, Casilla 114-D, Santiago 1, Chile. Phone: (56-2) 686-2865. Fax: (56-2) 222-5515. E-mail: mboric@genes.bio.puc.cl

The specific substrate for pepsanurin is vulnerable to endogenous proteases, inasmuch human plasma fails to produce the anti-ANP activity after incubation for 48 h at 37°C prior to pepsin digestion (6). Similarly, control hydrolysates obtained by pepsin digestion of serum albumin do not affect the renal response to ANP (6, 9). In preliminary experiments, we found that pepsanurin contains significant amounts of a kinin-like material, as detected by RIA using a polyclonal antibody against bradykinin (BK), whereas this kinin-like material is absent in pepsin hydrolysates from pre-incubated plasma. These findings prompted us to test whether kinins may be the agents responsible for the anti-ANP activity elicit by pepsanurin. In support of this possibility, we showed that the iv administration of low doses of BK (0.5-0.75 nmol/kg) 3 min before ANP, block the diuretic-natriuretic response induced by an ANP bolus (1 nmol/kg) (5). In addition, ANP blockade is induced by the ip or id injection of kinin-containing peptides of 16 or 18 aa, reportedly found after pepsin digestion of bovine kininogen (10). More recently, we showed that the 15 aa peptide Met-Lys-BK-Ser-Ser-Arg-Ile (from the human kininogen sequence, named PU-15) also inhibits ANP when injected either iv, id or ip, at doses of 0.25-0.4 nmol/kg (11). The anti-ANP effect of BK and pro-kinin peptides is completely inhibited by the specific kinin B2 receptor antagonist HOE-140 (5, 10-11). These reports clearly establish that administration of non-depressor doses of exogenous kinins can exert anti-ANP effects in absence of systemic hemodynamic changes. Furthermore, we showed that in anesthetized rats, the ANP excretory response is blunted by endogenous kinins accumulated during kininase II (angiotensin-converting enzyme, ACE) inhibition, an effect that is prevented by HOE-140 (5). Therefore, a kinin-mediated mechanism may explain the finding that ANP diuresis is blunted during ACE inhibition in humans (13, 23).

Despite the above cited evidence, until now there is no direct demonstration as to whether kinins, or kinin-like peptide mediates the antidiuretic activity of pepsanurin, and whether kininogens are the source of the active peptide(s) present in pepsanurin. To test these hypothesis, we investigated if the kinin receptor antagonist HOE-140 could prevent the anti-ANP effects of pepsanurin. Secondly, we analyzed the kinin content and anti-ANP activity of pepsin hydrolysates of fresh, pre-incubated, and kininogen-free plasma. Third, we assessed if BK, and pepsin hydrolysates of purified human kininogen, inhibit the activity of ANP on renal function when injected ip at doses comparable to those found in pepsanurin.

In addition, to gain a better knowledge about the mechanism of the pepsanurin, or kinin-induced inhibition of ANP activity, we determined the urinary excretion of cyclic GMP, the second messenger mobilized by ANP (7). Finally, we explored if the vasodilator and inflammatory action of kinins (3, 19) contribute to the anti-ANP effects by producing protein exudation and fluid shifts from plasma to the peritoneum. To address this possibility, we compared the effects of pepsanurin and BK with those of histamine, another agent known to induce local inflammation and vasodilation (18).

## MATERIALS AND METHODS

### *Animals and biological material*

Sprague-Dawley rats were bred and maintained at the University animal facilities. All studies were conducted following institutional and international policies for the welfare and well-being of animals, in compliance with the "Guiding Principles in the Care and Use of Laboratory Animals", endorsed by the American Physiological Society. Human plasma was obtained from the blood bank, *Hospital Clínico Universidad Católica*, within 8 h after being drawn and stored by standard techniques. Rat plasma was obtained by bleeding through the carotid artery, under pentobarbital anesthesia, using citrate to prevent clotting.

### *Reagents*

ANP 103-125 (Atriopeptin II, rat form), bradykinin (BK), cyclic GMP (cGMP), 2'-0-Succinyl-cGMP-Tyrosyl-Methyl-ester (S-cGMP-TME), bovine immunoglobulins (IgG), bovine serum albumin (BSA), histamine, papain, pepsin, and 4B activated sepharose were obtained from Sigma Chemical (St Louis, MO). Kinin antagonist HOE-140 (D-Arg-[Hyp$^3$, Thi$^5$, D-Tic$^7$, Oic$^8$]BK) was generously provided by Hoescht (Mainz, Germany). A specific antibody raised against cGMP was a gift from Dr M Currie, Searle Research & Development (Monsanto, St Louis, MO). All other reagents were of analytical grade from Merck (Darmstaad, Germany). Dialysis membranes of 3.5 kDa and 14 kDa cut-off (Spectra/Por 3 and 2), were obtained from Spectrum (Houston, TX).

### *Preparation of pepsanurin and control pepsin hydrolysates*

To prepare pepsanurin, fresh human or rat plasma was dialyzed overnight against distilled water (14 kDa cut-off membrane) and incubated with pepsin at pH 2.5 for 18 h at 37°C, as described (6, 9). The reaction was stopped by immersing the incubation mix in a boiling bath, for *ca.* 10 min, until the solution's temperature reached 80°C. After centrifugation at 3,000 rpm, the clear supernatant was aliquoted and stored at-30°C. Immediately before testing, the samples were thawed and neutralized with NaOH.

Inactive plasma hydrolysates were prepared by leaving human plasma samples in sterile conditions during 24 or 48 h at 37°C, prior to pepsin digestion, as described (6). To differentiate these preparations from pepsanurin, we termed them pre-incubated plasma hydrolysates (PIPH). Control bovine serum albumin hydrolysate (BSAH) was prepared by submitting a solution of 65 mg/mL BSA in 0.9% NaCl to the same procedure of dialysis followed by pepsin hydrolysis as for preparing pepsanurin.

### *Preparation of kininogen-free plasma and kininogen*

Kininogen-free plasma was prepared by passing human plasma through a carboxymethyl-papain (CM-papain) sepharose affinity chromatography column, as described (14). Briefly, CM-papain was prepared by overnight incubation of 100 mg papain with 10 mM iodoacetic acid, in 15 mL 1 mM EDTA, 2 mM dithiothreitol, pH 7.0. CM-papain was dialyzed against 0.1 M $NaHCO_3$, pH 9.0, and incubated overnight with 100 mL activated sepharose 4B with gently agitation. After blocking uncoupled sepharose groups with 0.1 M glycine, the resin was washed at pH 3.0 and 11.0, mounted in a 4.5 x 6 cm column and equilibrated by passage of 500 mL 50 mM sodium phosphate, 2 M NaCl, 1 mM benzamidine, 40 µg/mL polybrene, 1 mM EDTA, 0.2 mM PMSF, pH 7.5. Fresh human plasma samples were divided in two 60 mL aliquots. One aliquot was made 2 M NaCl, 10 mM benzamidine, 40 µg/mL polybrene, 2 mM EDTA, 0.2 mM PMSF and 0.2 µg/mL soybean trypsin inhibitor, and passed through the CM-papain column at 4 mL/min, followed by 100 mL equilibration buffer. The eluted fluid was collected in 4 mL fractions and the 15 fractions with higher protein concentration ($A_{280}$ elution plateau) were pooled as the kininogen-free (K-) plasma. The other 60 mL plasma aliquot was used as whole plasma (K+) control. The column was regenerated by eluting the attached kininogens with 50 mM $Na_3PO_4$, pH 11.5. Five matched whole-plasma (K+) and kininogen-free (K-) plasma samples were then independently submitted to dialysis and pepsin hydrolysis to prepare pepsanurin.

The eluted kininogen fraction was concentrated by ultrafiltration on Amicon PM-30 membrane and chromatographed on a semi-preparative HPLC ionic exchange DEAE column (Superformance 50-10, Lichrospher 1000, Merck) using a 0 to 0.5 M NaCl gradient in 10 mM sodium acetate, pH 5.5, to separate low (LMW) and high (HMW) molecular weight kininogens, as described (14). A 12 mL sample of each purified kininogen fraction was submitted to a time-controlled hydrolysis with pepsin, at pH 2.5, 37°C. The incubation was started by adding approximately 1 nmol pepsin per 10 nmol of estimated kininogen content

(per mL: 8 μg pepsin to 159 μg LMW kininogen, and 5 μg pepsin to 153 μg HMW kininogen) and was ended by adjusting the mixture to pH 6.1 and heating to 80°C for 5 min, followed by centrifugation at 10,000 g for 20 min. Production of active peptides was assessed at three times of hydrolysis: time zero (*i.e.*, immediately after adding pepsin), and after 1 or 4 h of incubation. As control for this series, we prepared a similar hydrolysate of 150 μg/mL BSA dissolved in 0.375 M NaCl, 10 mM sodium acetate, incubated with 8 μg/mL pepsin at pH 2.5, 37°C during 4 h.

### *Diuresis test*

The bioassay previously described by us was used (5-6, 9-11). Briefly, female rats (0.19-0.23 kg) were anesthetized with sodium pentobarbitone 65 mg/kg ip. Polyethylene cannulas were introduced in the trachea, left jugular vein, right femoral artery and left femoral vein. A constant infusion of 20 μL/min isotonic glucose solution was maintained via the jugular vein. Carotid arterial pressure was monitored with a Statham transducer connected to a Grass polygraph. Data reported correspond to mean arterial pressure. A silastic catheter was placed in the bladder through the urethra and urine was collected during ten periods of 20 min each. Three 20-min urine collection periods were allowed as baseline. At the start of period 4, the animals received an iv bolus of ANP, named S1, which consisted of 0.5 μg ANP (*ca.* 1 nmol/kg) dissolved in 0.1 mL of isotonic glucose. Under our experimental conditions, the diuretic response to ANP is largely completed within 20 min. Two 20-min collection periods were allowed for recovery. At the beginning of period 7, each rat was injected ip with either vehicle, pepsanurin, or other hydrolysates, BK, or histamine, or a combination of them as explained below. In all cases, Krebs solution was used as to complete a final volume of 10 mL/kg. A second iv ANP bolus (identical to the first bolus and referred to as S2), was given at the start of period 9, *i.e.*, forty min after the ip injection of the selected test substance. In some protocols BK receptors were blocked by injecting 5 μg/kg HOE-140 in a 0.1 mL iv bolus, 10 min before the ip injection of the test substance. In separate experiments we determined that this dose of BK antagonist was able to block the hypotension induced by 1-2 μg BK for a 2-h period.

### *Experimental protocols and doses*

The first protocol was designed to test the effect of pepsanurin and HOE-140 on ANP induced diuresis and saluresis. Six to ten animals were used in each experimental group. Three groups of rats were injected with either Krebs solution, or 1.0 or 2.5 mL/kg of rat pepsanurin. Three separate groups received the same doses of vehicle or rat pepsanurin, but they were previously injected with 5 μg HOE-140 iv.

The second protocol was designed to gain insight about the molecular weight and nature of the active components of pepsanurin. To this end, aliquots of human pepsanurin were dialyzed overnight at 4°C against Krebs solution using membranes of 3.5 or 14 kDa MW cut-off. The retained material was assayed for protein and immunoreactive kinin content, and for its ability to inhibit ANP, with or without HOE-140 pretreatment.

The third protocol was designed to determine if kininogens were the substrate for the active component(s) of pepsanurin. To this end, we compared the immunoreactive-kinin content and the anti-ANP effect of pepsin hydrolysates obtained from whole (K+) and kininogen-free (K-) plasma. Five matched K+ and K- plasma samples were tested (5 mL/kg) in four to five rats each, for a total of 22 determinations in each group. In addition, we analyzed the kinin content, and anti-ANP activity of purified LMW and HMW kininogen submitted to zero (non-hydrolysed), 1 or 4 h of pepsin hydrolysis in acid conditions. In this series, animals were injected ip with 0.25 mL of kininogen, or BSA, hydrolysates (4-7 rats per group).

The fourth protocol was designed to test whether or not ip injection of BK blocks the renal actions of ANP. Bradykinin doses of 2.5, 5, 10, and 20 μg/kg were chosen

because are comparable to the amount of immunoreactive kinin material found in pepsanurin samples. Larger doses of BK (50-100 μg / kg) induced transient systemic hypotension, thus precluding a fair testing of ANP-induced diuresis (data not shown).

The fifth protocol was designed to measure urinary cGMP excretion to gain insights into the mechanism of action of pepsanurin and/or BK inhibition of ANP renal actions. Three groups of rats were injected with 5 mL/kg of either BSAH, human pepsanurin, or BSAH plus 5 μg/kg BK. In this series, the urine was collected on ice and quickly frozen after taking aliquots for Na and K measurements (5).

The objective of the sixth protocol was to test for possible unspecific antidiuretic effects of osmotic and/or inflammatory actions of the peptidic material and/or kinins introduced in the peritoneum. Three groups of rats received respectively (per kg weight) 5 mL BSAH, 5 mL BSAH plus 5 μg BK, and 5 mL BSAH plus 15 μg histamine. The histamine dose was chosen after preliminary trials because it was the largest dose that did not significantly reduced mean arterial pressure. At the end of the experiment, the animals were killed with KCl and the amount of free fluid in the peritoneal cavity was aspirated and measured.

### *Determinations*

Diuresis was measured gravimetrically. Sodium and potassium excretion were determined in a Eppendorf flame spectrophotometer. Proteins were determined by the method of Lowry. BK was measured using a radioimmunoassay (RIA) as described (4). Briefly, aliquots of pepsanurin or control samples were appropriately diluted and incubated overnight at 4°C with a polyclonal anti-BK antibody, in the presence of 10,000 cpm $^{125}$I-[Tyr$^{8}$]-BK, used as tracer, in 0.5 mL final volume. The amount of free tracer was separated by adding dextran-coated charcoal and centrifugation. Free tracer counts were measured in a gamma counter (LKB-Wallac 1270, Turku, Finland) equipped with an automatic RIA program. Authentic BK (10-150 pg/tube ) was used as standard.

Urinary cGMP was determined in a RIA using $^{125}$I-S-cGMP-TME as tracer (5). Briefly, urine samples were thawed and diluted with RIA buffer (0.05 mol/L sodium acetate, pH 6.2). Aliquots (0.1 mL) were acetylated by adding 10 μL of triethylamine : acetic anhydride (2:1) and stirring. The samples were incubated overnight at 4°C, in 0.5 mL final volume, containing anti-cGMP antibody diluted 1:40,000 and 13,000 cpm of $^{125}$I-S-cGMP-TME. Similar tubes containing 10-1,000 fmol cGMP were used for the standard curve. After the incubation, 200 μg IgG was added and the antibody was precipitated in 50% ammonium sulfate and centrifuged. Bound counts were detected in the gamma counter for determination of cGMP content.

### *Data analysis*

Animals whose arterial pressure differed in, or more than, 15 mm Hg between the moment they receive the first and second ANP bolus were excluded from the analysis. Similarly, animals whose first response to ANP was in the upper and lower 5 percentile were not included in the study. The analysis was conducted in 210 out of 231 successfully completed experiments. As appropriate, some results are presented as the ratio between the second and first response to ANP (S2/S1 response ratio). In one table we show the baseline values preceding both ANP responses as well. Paired Student's *t* tests were used to assess differences between S1 and S2 within groups. To compare the potency of ANP inhibition among treatments, S2/S1 response ratios were submitted to arcsin transformation and analyzed using either unpaired Student's *t* test, or one-way ANOVA followed by the Newman-Keuls test. Unpaired Student's *t* tests with tables for multiple comparisons against a single control (12) were used in the BK dose-response curve.

## RESULTS

### *Renal excretory actions of ANP and their inhibition by Pepsanurin*

The injection of 0.5 μg ANP increased diuresis by 5-10 fold, sodium excretion by 50-100 fold, and potassium excretion by 2-3 fold (Figs 1, 2, Table I). These urinary changes were accompanied with a slight but significant reduction in mean arterial pressure (5-10 mm Hg; $p<0.05$ *vs* before ANP, paired Student's *t* test). All these effects waned and tended to vanish during the first 20 min collection period after the ANP injection. In control rats, the second response to ANP was greater than the first, giving typical S2/S1 response ratios between 1.2 and 1.4 for each of the excretory parameters studied (Fig 1). Mean arterial pressure was not different between S2 and S1. In contrast, animals injected with rat pepsanurin showed a significant reduction in volume, sodium and potassium excretion during the second ANP response, without evidencing changes in mean arterial pressure (Fig 1). The inhibition of ANP-induced natriuresis was proportional to the dose of pepsanurin.

Human pepsanurin both, crude or after partial purification through 3.5 kDa dialysis membranes, also inhibited the renal effects of ANP (Table I). Prolonged dialysis of human pepsanurin across a 3.5 kDa cut-off membrane resulted in loss of about half the total peptidic content (from 58.5 to 33.0 mg/mL), and a similar degree of reduction in the content of kinin-like material (from 333 to 129 ng BK/mL in this batch). It is noteworthy that the material retained by the 3.5 kDa cut-off membrane inhibited the diuresis and natriuresis induced by ANP in proportion to its kinin-like content (Table I). In contrast, dialysis with a 14 kDa cut-off membrane produced loss of 95% of the kinin-like material, accompanied by lost of the anti-ANP activity (Table I). In parallel trials, the recovery of authentic BK after dialysis in 3.5 kDa membranes was less than 1% if BK was dissolved in buffer alone, and less than 5% if BK was dissolved in buffer containing 65 mg/mL BSAH.

### *Blockade of the anti-ANP effect of pepsanurin by HOE-140.*

Treatment with 5 μg HOE-140 (iv) prior to the second ANP bolus did not modify the renal response to the atrial hormone (Fig 2). However, treatment with the kinin receptor antagonist completely blocked the anti-ANP effects of 1.0 mL/kg rat pepsanurin, and significantly reduced the anti-ANP effect of rat pepsanurin at 2.5 mL/kg (Fig 2). The efficacy of B2 receptor blockade attained with this dose of HOE-140 was confirmed by the lack of hypotensive effect of a 1 μg BK iv bolus given after completion of the collection periods, *i.e.*, 90 min after HOE-140 injection. Before treatment with HOE-140, BK induced a drop of 26.9 ± 3.2 mm Hg lasting 40.8 ± 2.3 s; after HOE-140, the effect of BK was a fall of 4.4 ± 1.8 mm Hg, lasting 7.6 ± 2.6 s ($p<0.0001$, paired Student's *t* test, n=9).

Pretreatment with HOE-140 also abolished the anti-ANP effects of dialyzed human pepsanurin (Table I), indicating that the active peptide(s) retained by the 3.5 kDa dialysis membrane also required activation of B2 receptors to exert its anti-ANP activity.

### *Kinin-like material and anti-ANP activity in plasma hydrolysates.*

Pepsanurin obtained by hydrolysis of fresh rat plasma, contained 919 ± 51 ng/mL of kinin-like material as detected by the RIA for BK (n=6). Pepsanurin obtained from fresh human plasma contained 699 ± 153 ng/mL of immunoreactive kinin (n=10). The kinin-like material decreased to 26 ± 13 ng/mL in matched human plasma samples hydrolyzed with pepsin after 24 h of pre-incubation at 37°C (n=5). Kinin immunoreactivity was below the detection threshold (<2 ng/mL) in human plasma hydrolyzed with pepsin after 48 h of pre-incubation at 37°C (n=6). We have previously reported that both human and rat pepsanurin inhibit the diuresis induced by ANP, whereas matched samples from pre-incubated plasma did not inhibit ANP renal effects (6, 9).

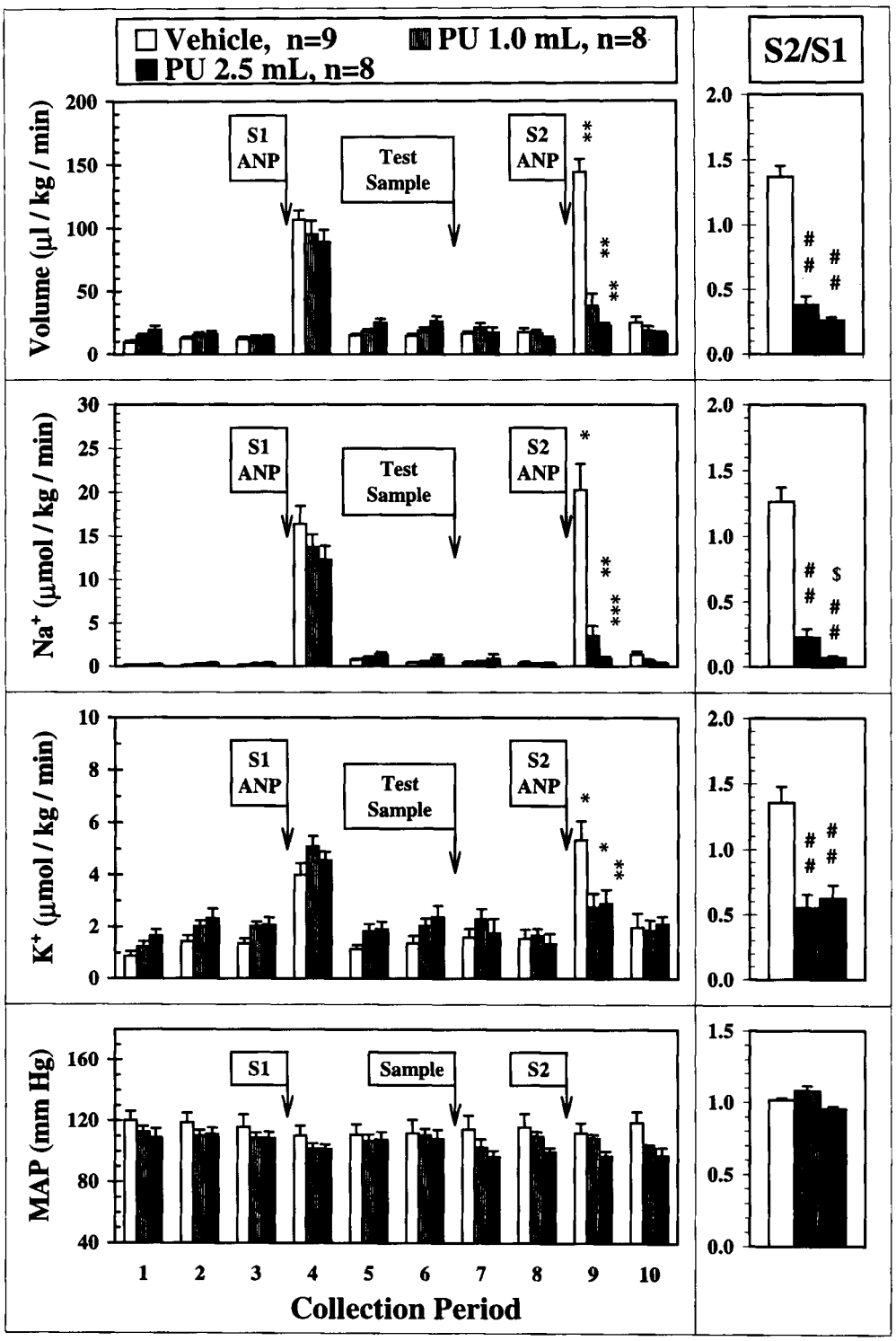


**Fig 1.** Inhibition of ANP-induced excretion by rat pepsanurin. Time course of urinary volume, sodium and potassium excretion, and mean arterial pressure (MAP), studied in 3 groups of anesthetized rats during 10 data collection periods of 20 min each. All rats received 0.5 µg ANP iv as stimulus at start of periods 4 and 9 (S1 and S2, arrows). At beginning of period 7 (test sample, arrow), rats received ip injections of either Krebs solution (vehicle) (open bars), 1.0 (vertically striped bars) or 2.5 (filled bars) mL/kg of rat pepsanurin (PU), injected volumes being completed to 10 mL/kg with Krebs solution in all groups. Means ± SEM. * $p<0.05$; ** $p< 0.001$; *** $p<0.0001$ between S2 and S1, paired Student's *t* tests. Right side panels, S2/S1 ANP response ratios. ## $p<0.001$ *vs* vehicle; \$ $p<0.05$ *vs* 1.0 mL PU; ANOVA and Newman-Keuls tests. $F(2,22) = 98.8$, $p< 2^{-11}$ for volume; $F(2,22) = 103.9$, $p< 2^{-11}$ for sodium; and $F(2,22) = 14.8$, $p< 1^{-4}$ for potassium.

**Table I**

Inhibition of atrial natriuretic peptide excretory action by human pepsanurin: effects of dialysis and HOE-140

| | PRE-TREATMENT BASAL | PRE-TREATMENT ANP (S1) | POST-TREATMENT BASAL | POST-TREATMENT ANP (S2) | RESPONSE RATIO (S2/S1) |
|---|---|---|---|---|---|
| **Crude Pepsanurin** # (n=6) | | | | | |
| Volume | 21.6 ± 5.6 | 146.4 ± 20.2 | 15.9 ± 2.2 | 68.1 ± 14.5** | 0.466 ± 0.091 |
| Sodium | 0.23 ± 0.09 | 25.21 ± 4.03 | 0.38 ± 0.13 | 8.91 ± 2.77** | 0.378 ± 0.127 |
| Potassium | 2.81 ± 0.64 | 6.48 ± 0.73 | 2.69 ± 0.71 | 4.97 ± 0.99 | 0.781 ± 0.128 |
| MAP | 127 ± 5.3 | 115 ± 4.1 | 131 ± 5.7 | 123 ± 5.7 | 1.070 ± 0.056 |
| **Retained in 3.5 kDa** ## (n=7) | | | | | |
| Volume | 13.4 ± 3.6 | 104.3 ± 21.0 | 14.4 ± 1.7 | 64.2 ± 17.3** | 0.606 ± 0.116 |
| Sodium | 0.23 ± 0.11 | 19.34 ± 4.06 | 0.41 ± 0.14 | 9.24 ± 3.06** | 0.465 ± 0.149 |
| Potassium | 2.26 ± 0.87 | 3.67 ± 0.42 | 2.41 ± 0.35 | 4.67 ± 0.63 | 1.347 ± 0.174 |
| MAP | 119 ± 4.7 | 112 ± 3.3 | 115 ± 4.6 | 108 ± 5.7 | 0.963 ± 0.040 |
| **Retained in 3.5 kDa ## plus HOE-140** (n=7) | | | | | |
| Volume | 10.5 ± 0.7 | 111.9 ± 14.5 | 16.6 ± 1.6 | 137.9 ± 19.6* | 1.248 ± 0.135 |
| Sodium | 0.17 ± 0.03 | 20.07 ± 2.42 | 0.49 ± 0.15 | 20.96 ± 2.88 | 1.057 ± 0.127 |
| Potassium | 2.02 ± 0.35 | 5.16 ± 0.65 | 2.38 ± 0.22 | 6.48 ± 0.93* | 1.295 ± 0.141 |
| MAP | 114 ± 4.6 | 106 ± 3.5 | 129 ± 6.5 | 118 ± 5.1* | 1.121 ± 0.052 |
| **Retained in 14 kDa** ### (n=5) | | | | | |
| Volume | 8.8 ± 0.8 | 79.1 ± 12.6 | 15.4 ± 0.8 | 138.5 ± 21.3* | 1.978 ± 0.452 |
| Sodium | 0.14 ± 0.04 | 14.68 ± 2.95 | 0.34 ± 0.12 | 22.30 ± 3.74 | 1.966 ± 0.550 |
| Potassium | 1.35 ± 0.42 | 3.98 ± 0.70 | 3.27 ± 0.71 | 8.17 ± 1.18** | 2.296 ± 0.456 |
| MAP | 114 ± 4.3 | 107 ± 3.7 | 125 ± 4.2 | 118 ± 5.8* | 1.101 ± 0.032 |

# 0.5 mL containing 29.3 mg protein and 166 ng BK equivalent; ## 1.0 mL with 33.0 mg protein and 129 ng BK equivalent; ### 1.0 mL with 14.3 mg protein and 13 ng BK equivalent. Volume in μL/kg/min; sodium and potassium in μEq/kg/min; mean arterial pressure (MAP) in mm Hg. * $p < 0.05$; ** $p < 0.01$ *vs* first ANP response; paired Student's *t* tests.

***Effects of kininogen depletion.***

Passage of fresh human plasma through the CM-papain affinity column produced an almost complete absorption of kininogens. Pepsanurin obtained from whole (K+) plasma contained 75.6 ± 3.8 mg/mL protein and 526 ± 184 ng/mL kinin-like material (n=5), whereas matched hydrolysates from kininogen-free samples (K-) contained 61.8 ± 3.7 mg/mL protein and only 3.1 ± 1.5 ng/mL immunoreactive-kinin (n=5). The ratio kinin / protein dropped two hundred-fold, from 699 ± 247 x $10^{-4}$ in K+ to 5.0 ± 2.6 x $10^{-4}$ in K-. As observed in Figure 3, while pepsanurin (K+ plasma hydrolysates) inhibited ANP-induced urinary excretion of volume, sodium, and potassium by 67 ± 4 %, 80 ± 5 % and 40 ± 10 % respectively, K- plasma hydrolysates were completely inert. Basal excretion, and the first response to ANP were similar in both groups, therefore, only S2/S1 response ratios are shown.

***Effect of purified kininogens.***

Injection of non-hydrolysed LMW or HMW kininogen did not modify the diuretic effects of ANP (Table II). However, incubation of both plasma substrates with pepsin at acid pH resulted in the rapid production of material with the same inhibitory effect as pepsanurin (Table II). With the single dose used in this study, effective inhibition of ANP was obtained after 1 or 4 h hydrolysis of both LMW and HMW kininogen.

***Inhibition of ANP-induced diuresis and saluresis by ip BK.***

The ip administration of BK (2.5-20 μg/kg) 40 min prior to the second ANP bolus,

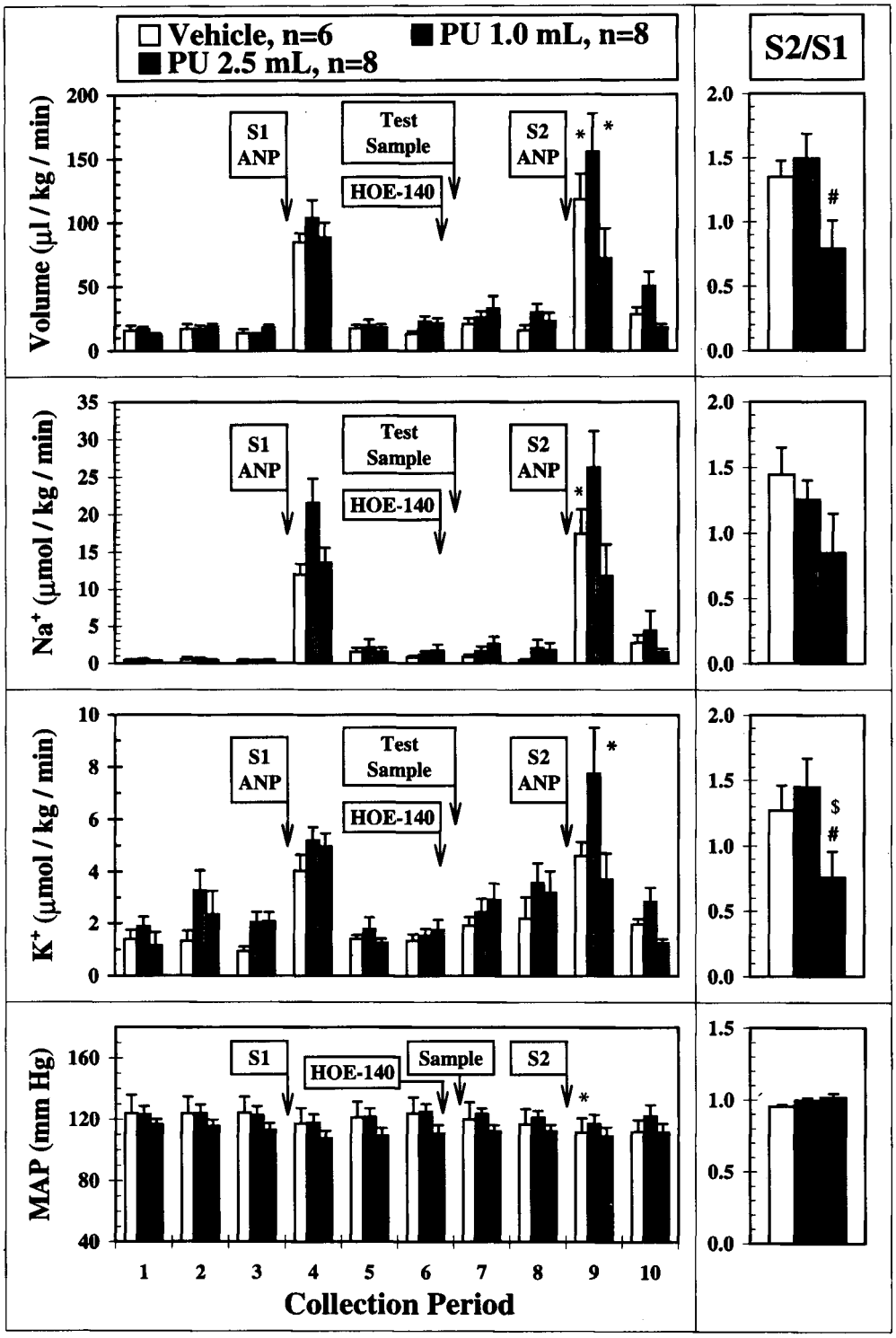


**Fig 2.** Blockade of anti-ANP effect of rat pepsanurin by HOE-140. Time course and S2/S1 response ratios for volume, sodium and potassium excretion, and mean arterial pressure (MAP), in three groups of rats injected with ANP and rat pepsanurin or vehicle as shown in Fig 1. In addition, all rats received 5 µg iv of B2 receptor blocker HOE-140 5-min before beginning of period 7, *i.e.*, 5-min before injection of test substance. # $p<0.05$ *vs* vehicle S2/S1; $ $p<0.05$ *vs* 1.0 mL pepsanurin; ANOVA and Newman-Keuls tests. All other symbols and notations as in Fig 1. $F(2,19) = 4.95$, $p< 0.02$ for volume; $F(2,19) = 3.09$, $p< 0.07$ for sodium; $F(2,19) = 4.44$, $p< 0.03$ for potassium.

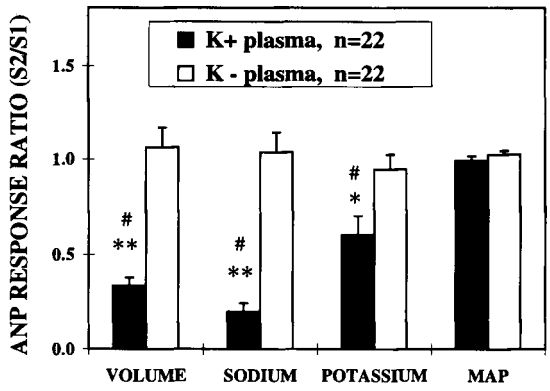


**Fig 3.** Lack of effect of kininogen-free plasma hydrolysates. Comparative effects of pepsanurin obtained from whole human plasma (K+ plasma) and kininogen-free plasma (K- plasma) on ANP response ratios (S2/S1) for urinary volume, sodium, and potassium excretion, and mean arterial pressure. Bars show the pooled results from five matched K+ and K- human plasma pepsin hydrolysates injected (5 mL/kg ip) in 4 or 5 rats each. Details as in Fig. 1. * $p<0.05$, ** $p<0.0005$ *vs* K- plasma, unpaired Student's *t* test; # $p<0.0005$ S2 *vs* S1, paired Student's *t* test.

produced a dose-related inhibition of the diuresis, natriuresis and kaliuresis induced by the atrial hormone, in absence of any detectable change in mean arterial pressure (Fig 4). Basal renal excretion and mean arterial pressure did not differ significantly among all these experimental groups. The response to the first administration of ANP was also similar among all groups, therefore only S2/S1 response ratios are presented for simplicity (Fig 4).

### *Effects of pepsanurin and BK on ANP-induced cGMP urinary excretion.*

In general, urinary cGMP followed the same pattern as volume excretion, increasing about 5-10 times after the first ANP bolus (Fig 5). Similarly, in BSAH injected rats, cGMP excretion was slightly greater during the S2 response as compared to S1. In contrast, cGMP urinary excretion was markedly attenuated during the S2 response in animals injected with either, pepsanurin or BK plus BSAH. The reduction in cGMP excretion was proportional to the reduced rate of diuresis. Interestingly, urinary cGMP was greater than baseline during collection period 10, particularly in those groups showing a blunted ANP response. This observation suggests a delayed production or release of this intracellular mediator.

## Table II

Immunoreactive kinin content and anti-ANP activity of pepsin hydrolysates of purified human kininogens

| Hydrolysis time (h) | Protein (µg/mL) | IR-kinins (ng/mL) | ANP response ratio (S2/S1) # | | | | |
|---|---|---|---|---|---|---|---|
| | | | volume | sodium | potassium | MAP | n |
| **HMW kininogen** | | | | | | | |
| 0 | 120 | 6 | 1.177±0.194 | 1.078±0.162 | 1.347±0.222 | 0.933±0.032 | 4 |
| 1 | 130 | 71 | 0.575±0.172* | 0.464±0.199* | 0.639±0.137* | 1.038±0.037 | 7 |
| 4 | 130 | 104 | 0.477±0.117* | 0.077±0.036** | 0.545±0.161 | 0.997±0.085 | 4 |
| **LMW kininogen** | | | | | | | |
| 0 | 175 | 0 | 0.815±0.121 | 0.795±0.261 | 1.185±0.248 | 1.046±0.025 | 5 |
| 1 | 190 | 227 | 0.348±0.099*** | 0.153±0.085*** | 0.375±0.082** | 0.949±0.082 | 7 |
| 4 | 185 | n d | 0.438±0.028** | 0.108±0.048*** | 0.835±0.141 | 0.898±0.103 | 4 |
| **BSA** 4 | 160 | 0 | 1.424±0.273 | 1.340±0.241 | 1.267±0.165 | 1.015±0.061 | 5 |

0.5 µg ANP injected iv before (S1) and after (S2) ip injection of 0.25 mL kininogen or BSA hydrolysate per rat. Mean arterial pressure (MAP). n d = not determined; * $p<0.05$; ** $p<0.005$; *** $p<0.0005$, S2 *vs* S1; paired Students's *t* tests.

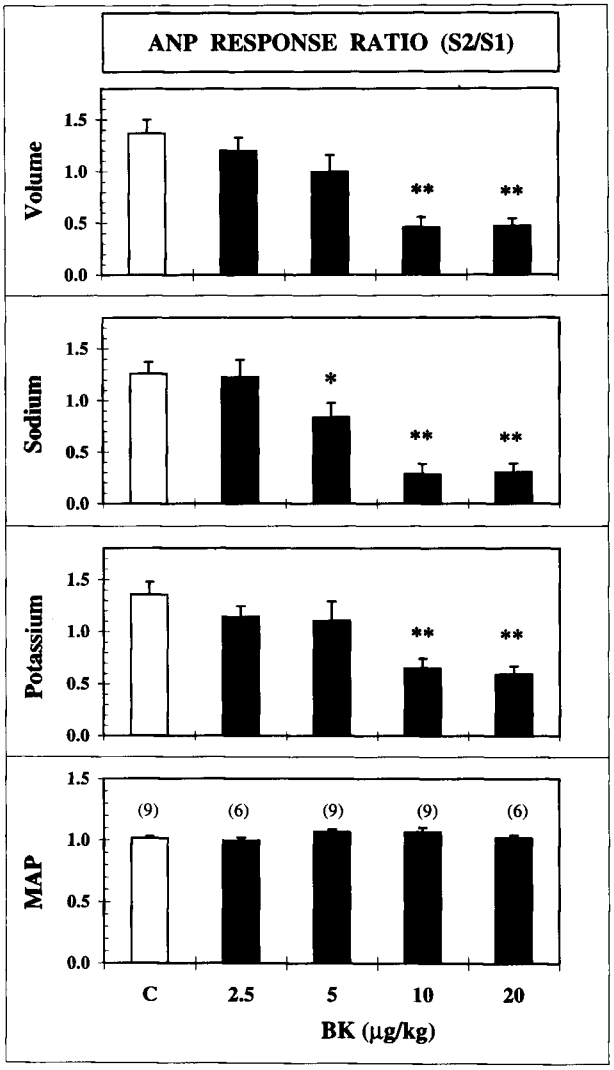


Fig 4. Inhibition of ANP-induced excretion by BK. ANP response ratios (S2/S1) for (from top to bottom) urinary volume, sodium excretion, potassium excretion and mean arterial pressure in five groups of rats that received either Krebs solution (control) or 2.5, 5, 10 and 20 µg/kg BK ip 40 min before S2. The experimental protocol as explained in Fig 1. * $p<0.05$, ** $p<0.005$ *vs* control S2/S1, unpaired Student's *t* test with tables for multiple comparisons.

### *Effects of albumin hydrolysates and histamine.*

The ip injection of BSAH in amounts similar to the protein content found in pepsanurin hydrolysates (330 mg in 5 mL/kg plus 5 mL/kg Krebs solution) did not affect the response to ANP (Fig 6). In BSAH injected rats S2/S1 response ratios were similar to those of controls injected with Krebs solution (Figs 1, 6). The addition of 10 µg/kg BK to BSAH produced a significant inhibition of ANP-induced diuresis and saluresis, again without variations in mean arterial pressure (Fig 6). In contrast to BK, the injection of histamine (15 µg/kg) plus BSAH, did not affect diuresis and natriuresis induced by ANP. Nevertheless, the amount of fluid accumulated in the peritoneal cavity of animals injected with BSAH plus histamine was almost twice that of animals injected with BSAH or BK plus BSAH (Fig 6).

## DISCUSSION

We demonstrate that kinins derived from kininogen are the chemical agents responsible for the inhibition of ANP renal actions induced by pepsanurin. This conclusion is supported by four findings: 1) the presence of immunoreactive kinin material in the active pepsanurin extracts, compared with its absence in inactive hydrolysates of pre-incubated plasma; 2) hydrolysates from kininogen-free plasma do not exert anti-ANP activity; 3) HOE-140, a specific B2 kinin receptor antagonist prevents the inhibitory action of human or rat pepsanurin on ANP-induced diuresis, natriuresis and kaliuresis; and 4) the ip administration of 5-20 µg/kg BK, or pepsin hydrolysates of purified kininogens, mimics the inhibitory effect of pepsanurin upon ANP renal excretion.

Our results indicate that pepsin hydrolysis of plasma kininogens produces peptides which contain the kinin sequence and are able to counteract ANP-induced diuresis by stimulation of B2 receptors. We had reported previously that pepsin hydrolysates of pre-incubated plasma maintained at 37°C for 24-48 h do not inhibit ANP (6, 9), and now we demonstrate that these hydrolysates do not contain kinin-like material, in contrast to active pepsanurin (pepsin hydrolysates of fresh plasma). In addition, plasma devoided of kininogens by separation in papain-sepharose affinity chromatography do not yield the active peptides when digested with pepsin. Taken together, these findings suggest that during prolonged incubation at

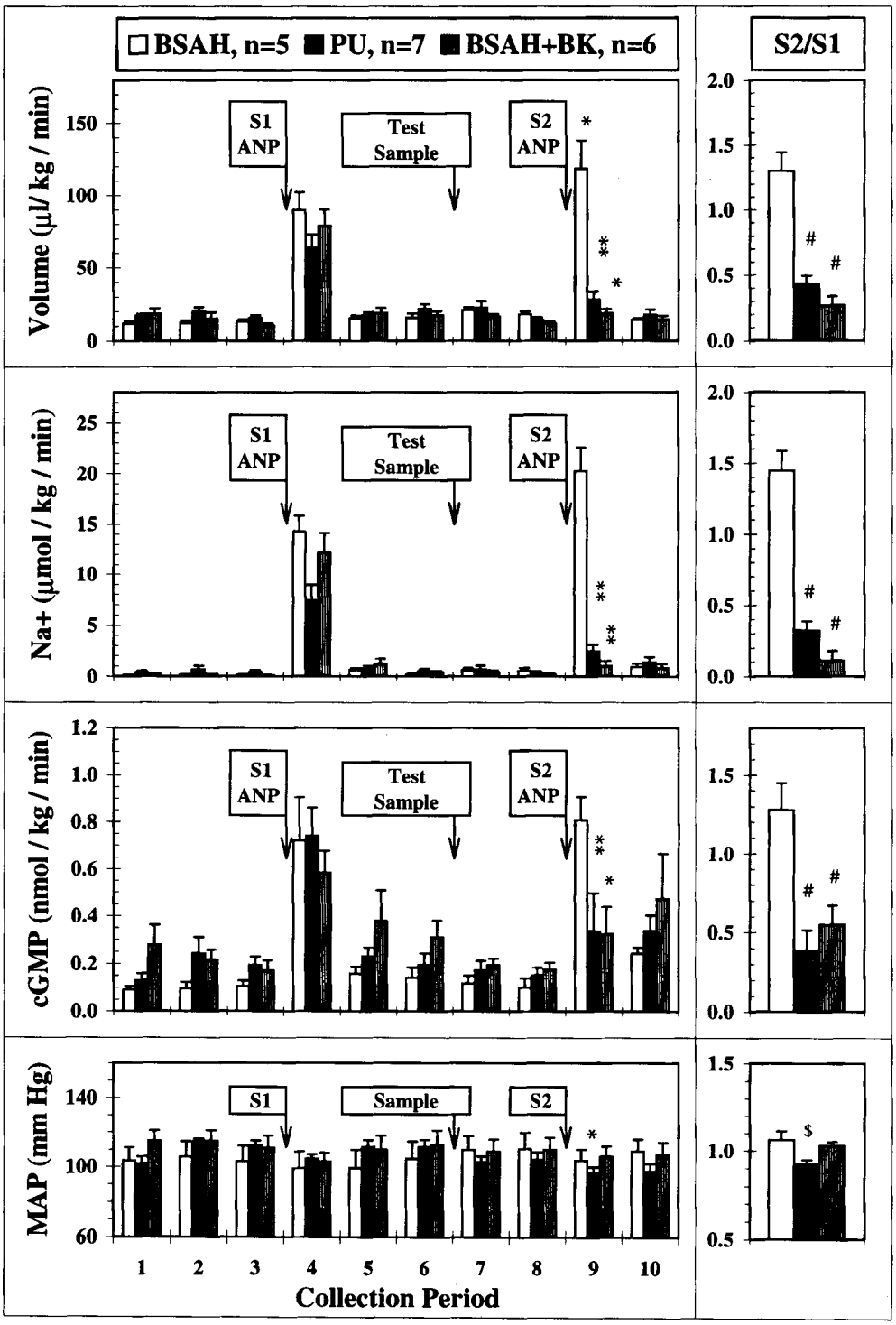


**Fig 5.** Effects of pepsanurin and BK on ANP-induced cyclic GMP excretion. Left panel: Time course of (from top to bottom) urinary volume, sodium excretion, cGMP excretion, and mean arterial pressure in three groups of anesthetized rats studied during 10 periods of 20 min each. All rats received 0.5 µg ANP iv as a stimulus at the start of periods 4 and 9 (S1 and S2, arrows). At the beginning of period 7 (test sample, arrow), the rats received ip injections of 5 mL/kg of either BSAH, or BSAH plus 10 µg/kg BK, or human pepsanurin (PU). Means ± SEM. * $p<0.05$; ** $p< 0.001$, between S2 and S1, paired Student's *t* test. Right panel: corresponding S2/S1 response ratios. # $p<0.05$ *vs* BSAH, $ $p<0.05$ *vs* the other two columns, ANOVA and Newman-Keuls test. $F(2,15) = 32.2$, $p< 4^{-6}$ for volume; $F(2,15) = 28.1$, $p< 1^{-5}$ for sodium; $F(2,15) = 12.7$, $p< 6^{-4}$ for potassium; $F(2,15) = 4.45$, $p< 0.03$ for arterial pressure.

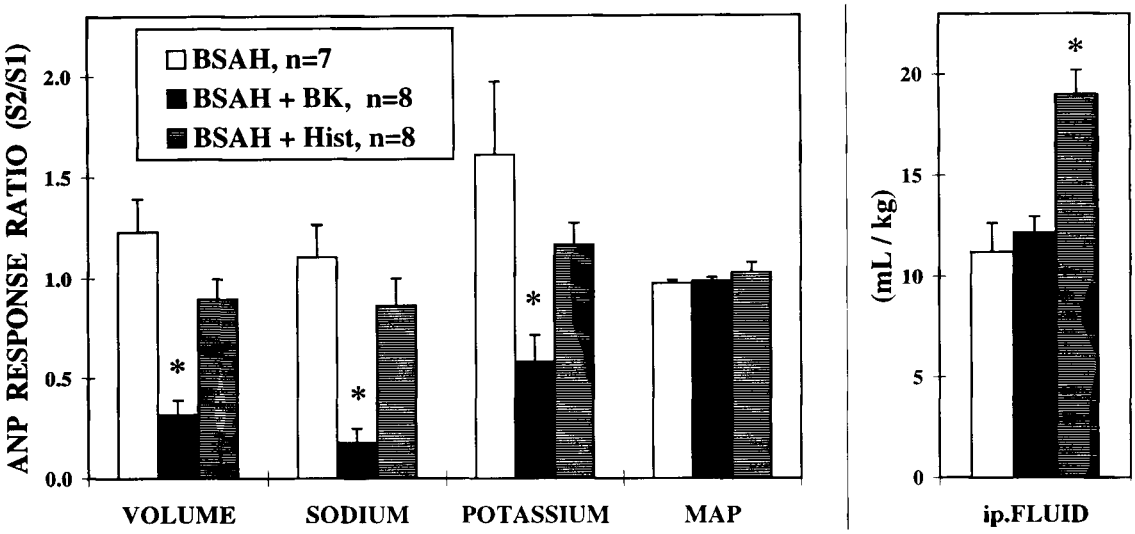


**Fig 6.** Effects of BSAH, BK and histamine on ANP-induced excretion, mean arterial pressure and peritoneal fluid accumulation. Left panel: response ratios (S2/S1) to iv injections of ANP before and after ip injections of either 5 mL/kg hydrolyzed bovine serum albumin alone (BSAH), or plus 10 µg/kg BK, or plus 15 µg/kg histamine. Right panel: volume of ip fluid recovered at end of experiment. * $p<0.05$ *vs* other two columns; ANOVA and Newman-Keuls tests. $F(2,20) = 16.6$, $p< 6^{-5}$ for volume; $F(2,20) = 14.9$, $p< 1^{-4}$ for sodium; $F(2,20) = 5.54$, $p< 0.02$ for potassium; $F(2,20) = 13.8$, $p< 2^{-4}$ for ip fluid.

37°C plasma kininogens are degraded to a degree that no active peptides are produced when incubated with pepsin.

Since the material retained after dialysis of human pepsanurin in 3.5 kDa membranes counteracts ANP-diuresis in proportion to its kinin-like content, we postulate that kininogen-derived kinin analogs, unable to diffuse through the 3.5 kDa dialysis membrane, are responsible for about half the anti-ANP activity of crude pepsanurin. BK itself, or other diffusible kinins such as kallidin or Met-Lys-BK (3) could account for the remaining anti-ANP activity of crude pepsanurin. We have shown recently that the peptide Met-Lys-BK-Ser-Ser-Arg-Ile (PU-15) (11) is very active to inhibit ANP, acting at equivalent doses when injected either iv or id. Other two BK-containing peptides of 16 and 18 aa, synthesized according to the sequences reported to appear after pepsin hydrolysis of bovine kininogen, also elicit the anti-ANP effect (10) although they are less potent than Met-Lys-BK-Ser-Ser-Arg-Ile. These, or other large-size kinins, must exert their effect by activation of B2 receptors, perhaps after conversion to BK in the living animal, as evidenced by HOE-140 blockade. Nevertheless, with the current data we cannot exclude the possibility that other peptides produced by pepsin hydrolysis of plasma kininogens may enhance or potentiate the actions of kinins.

### *Possible mechanisms of pepsanurin and BK inhibitory action upon ANP.*

We reported that pepsin hydrolysates of serum albumin, used as control for the bulk of non specific peptidic material do not inhibit ANP (6, 9-10). Now we extend this observation by demonstrating that the combined osmotic and inflammatory effects of inert peptides injected together with a vasodilator agent that increases protein exudation, such as histamine, are unable to elicit a significant inhibition of the ANP renal excretory effects. Because histamine plus BSAH induced a greater peritoneal fluid accumulation than pepsanurin, or BK plus BSAH, and because that fluid transfer did not alter ANP-induced renal excretion, we may rule out the concept that nonspecific inflammatory reactions and fluid shifts from plasma to peritoneum play a major role in the anti-natriuretic effect of either BK or pepsanurin.

We did not address directly the possibility that mesenteric vasodilation

induced by the ip administration of BK, or pepsanurin, may lead to reflex renal vasoconstriction contributing to the ANP blockade. Although measurements of renal and mesenteric blood flow would exactly settle this point, we think the ip administration of histamine may serve as an indirect control for the presumptive vasodilator effect in the mesenteric vascular bed. Three additional findings do not support the idea that redistribution of blood flow away from the kidney is a factor that may explain the anti-ANP effects. First, we have demonstrated inhibition of ANP diuretic effects after the intravenous administration of bradykinin (5), or Met-Lys-BK-Ser-Ser-Arg-Ile (11). It is unlikely that a particular vasodilation of the splanchnic circulation and a concomitant constriction of the renal circulation takes place under these conditions. Second, pepsanurin blocked ANP-induced diuresis in the isolated perfused rat kidney, where there is no possibility for blood flow redistribution to other organs (6). Third, urinary cGMP output is reduced in parallel to the blunted diuretic response observed after BK or pepsanurin suggesting a blockade of ANP transduction, rather than an effect secondary to a change in renal hemodynamics (7).

Because BK is also an algesic agent that stimulates nerve terminals (16), it may be argued that BK stimulates afferent pain fibers in the peritoneum which, in turn increase the sympathetic tone to the kidney (24). This suggestion is consistent with the report that enhanced renal nerves stimulation can prevent natriuresis induced by ANP (15). However, the same experimental findings discussed above, argue against a major involvement of the nervous system in the inhibition of ANP caused by BK or pepsanurin. The effects of pepsanurin in the isolated and denervated perfused kidney (6), as well as ANP inhibition after injection of kinins id (9-11) or iv (5, 11), suggest that kinins act directly on kidney structures where ANP promotes sodium excretion.

Our early attempts to block ANP activity by injecting or infusing pepsanurin iv in amounts that are effective when administered ip or id, were unsuccessful, particularly because crude pepsanurin preparations induce a state of unstable arterial pressure in the rat (unpublished observations). Similar negative results are observed if doses of 5-20 μg/kg BK are given iv at different times prior to ANP. These puzzling observations have been clarified in a previous study in which we reported that BK inhibits ANP renal actions when injected iv 3 min prior to ANP only at non-depressor doses within a narrow range of 0.5-0.8 μ/kg. In contrast, larger and lower BK doses are ineffective (5). A similar U-shaped inhibitory curve was obtained with Met-Lys-BK-Ser-Ser-Arg-Ile (11). These findings support the concept that the injection of pepsanurin (6, 9, this study), or pro-kinins (10-11), in the peritoneum or the intestinal lumen, produce a slow absorption and/or transformation of pro-kinins that reach a precise intrarenal kinin concentration required to elicit the anti-ANP effect. The same reasoning may explain the ten-fold difference found between the effective dose range of BK given ip (5-20 μg/kg, 40 min prior to ANP) or iv (0.5-0.8 μg/kg, 3 min prior to ANP). In the present study we did not found a U-shaped dose-response curve for ANP inhibition *vs* ip BK, probably because we did not test a dose large enough to exceed the inhibitory range, or perhaps because there is a delayed or saturated BK absorption through the peritoneum. These concepts have to be confirmed by timed measurements of circulating and intrarenal kinin concentration after kinin injection through the different routes.

In this report, we demonstrate that inhibition of ANP by either pepsanurin or BK is associated with a decreased urinary excretion of cGMP, favoring the view that there is an intrarenal mechanism affecting ANP interaction with its bioactive, guanylyl-cyclase receptors (7, 17). At this moment we cannot resolve the mechanism for the kinin-mediated inhibition of renal ANP action, but it is worth mentioning that, the diuretic, natriuretic and cGMP excretory effects of ANP are also blocked by endogenous kinins, as demonstrated in rats treated with kininase-II inhibitors (5).

An important question arising from our experiments is the physiological meaning of the kinin blunting of ANP-induced increase in renal excretion, particularly since local kinin production by the renal kallikrein-kininogen system has been proposed to play an important role in promoting natriuresis (22). However, kinins do not seem to mediate the excretory effects of ANP (1, 5). Our reports that pepsanurin (9), or kinins (10-11) produced a striking inhibitory effect on ANP renal actions, when introduced into the duodenal lumen in nanomolar doses, provide support for a role of intestinal-borne kinins as modulators of ANP-induced renal excretion. Pepsanurin was described in a historical context when pepsin was used as a probe to detect bioactive peptides that could be released enzymatically from plasma precursors. Recently, we have advanced the concept that a peptide generated in the digestive tract may serve as a regulatory signal from the intestine to the kidney, as part of fluid homeostasis during the prandial cycle (9-10). ANP alters fluid transport in the intestine, favoring net fluid secretion to the lumen (2, 20, 21). Therefore, circulating ANP may facilitate fluidity of intestinal content during digestion, provided that its renal excretory action is blocked. On the other hand, the kallikrein-kinin system is widely expressed in the intestinal tract and secretory glands (3). Thus, we speculate that kinins released during digestion by pancreatic or salivary kallikrein, as well as by other proteases including pepsin, may act as signals to delay renal sodium and water excretion, however the physiological relevance of this putative mechanism requires further evaluation.

## ACKNOWLEDGMENTS

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