

Atrial Natriuretic Peptide₍₃₁₋₆₇₎ Inhibits Na⁺ Transport in Rabbit Inner Medullary Collecting Duct Cells

Role of Prostaglandin E₂

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Abstract

Atrial natriuretic peptide (ANP)₍₃₁₋₆₇₎, a portion of the atrial peptide prohormone, circulates in humans, and its plasma level varies with atrial pressure. Like the more widely studied carboxy-terminal fragment ANP₍₉₉₋₁₂₆₎, ANP₍₃₁₋₆₇₎ stimulates natriuresis and diuresis. We examined the mechanism of this natriuresis by measuring the effects of ANP₍₃₁₋₆₇₎ on Na⁺ transport in cells of the rabbit inner medullary collecting duct (IMCD). ANP₍₃₁₋₆₇₎ (10⁻⁸ M) caused a 26±4% inhibition of oxygen consumption (QO₂); half-maximal inhibition occurred at 10⁻¹¹ M, suggesting a physiologic effect. This effect was not additive with either ouabain or amiloride, suggesting that it reflected inhibition of Na⁺ transport-dependent QO₂. ANP₍₃₁₋₆₇₎ reduced the amphotericin-induced stimulation of QO₂, consistent with inhibition by this peptide of the Na⁺-K⁺-ATPase. In addition, ANP₍₃₁₋₆₇₎ reduced ouabain-sensitive ⁸⁶Rb⁺ uptake under V_{max} conditions. Several lines of evidence indicated that PGE₂, a known endogenous IMCD Na⁺-K⁺-ATPase inhibitor, mediates pump inhibition by ANP₍₃₁₋₆₇₎. Thus, ANP₍₃₁₋₆₇₎ inhibits Na⁺ transport by inhibiting the Na⁺-K⁺-ATPase of IMCD cells, an effect mediated by the generation of PGE₂. (*J. Clin. Invest.* 1992. 89:1411-1417.) Key words: Na⁺-K⁺-ATPase • renal medulla • Na⁺ • Pro-ANP • atrial natriuretic peptide • collecting duct

Introduction

Early studies of the potent natriuretic and diuretic properties of atrial natriuretic peptides used a crude saline extract from homogenized atria which, when injected into assay rats provoked striking natriuresis, diuresis, and hypotension (1-3). Subsequent studies soon demonstrated that atria secrete a prohormone, atrial natriuretic peptide (ANP)₍₁₋₁₂₆₎,¹ from which the carboxy-terminal 28 amino acid ANP₍₉₉₋₁₂₆₎ is rapidly cleaved (4-6). Because the most readily identifiable circulating product of ANP₍₁₋₁₂₆₎ was ANP₍₉₉₋₁₂₆₎ (also known as ANP₍₁₋₂₈₎), or sim-

ply ANP), virtually all subsequent studies were directed towards defining the action of this carboxy-terminal fragment (4). Little attention was paid to the other product of cleavage, ANP₍₁₋₉₈₎, and its derivative peptides. The original infusion studies with atrial extracts presumably involved ANP₍₁₋₉₈₎ and fragments thereof, as well as ANP₍₉₉₋₁₂₆₎, and, subsequently, Kangawa et al. showed that infusion of intact ANP₍₁₋₉₈₎ had natriuretic effect (5, 7).

More recent evidence suggests that a fragment of ANP₍₁₋₉₈₎, ANP₍₃₁₋₆₇₎, also circulates in plasma (8, 9). Antibodies raised against synthetic ANP₍₃₁₋₆₇₎ and specific for this peptide identified a circulating peptide of molecular weight similar to that of synthetic ANP₍₃₁₋₆₇₎ (molecular mass ~ 4,000 D). This circulating peptide coeluted with synthetic ANP₍₃₁₋₆₇₎ on reverse phase HPLC (8, 9). Furthermore, this same peptide circulates at a concentration greater than ten times that of ANP₍₉₉₋₁₂₆₎, and, as with ANP₍₉₉₋₁₂₆₎, the concentration of the peptide increases in the setting of increased right atrial pressure (e.g., head-out water immersion [HOWI] or congestive heart failure) (9, 10). Finally, infusion of synthetic ANP₍₃₁₋₆₇₎ into rats proves it to be natriuretic and diuretic, similar in potency to ANP₍₉₉₋₁₂₆₎ (11). Since the natriuretic effect of ANP₍₃₁₋₆₇₎ was not accompanied by any change in glomerular filtration rate (GFR), the peptide, like ANP₍₉₉₋₁₂₆₎, must have a tubule site of action (11). Thus, there is strong evidence that atrial regulation of renal Na⁺ excretion may not be mediated solely through the carboxy-terminal ANP₍₉₉₋₁₂₆₎ fragment of the ANP₍₁₋₁₂₆₎ molecule, but also via the product of the cleaved ANP₍₁₋₉₈₎, namely ANP₍₃₁₋₆₇₎.

We and others have previously shown that ANP₍₉₉₋₁₂₆₎ binds to specific cell-surface guanylate cyclase-linked receptors, leading to increased cGMP generation, which in turn reduces the open time of luminal plasma membrane sodium channels (12-15). Inhibition of these Na⁺ channels is believed to cause reduced Na⁺ reabsorption, leading to natriuresis (3, 12, 15). We have, therefore, now examined the effect of ANP₍₃₁₋₆₇₎ on Na⁺ transport in these cells, and determined the mechanism by which this ANP fragment acts on the inner medullary collecting duct (IMCD) cell to exert its natriuretic effect.

Methods

Tissue preparation. Fresh suspensions of IMCD cells were prepared as described previously (13, 16). Anesthetized rabbits were decapitated and exsanguinated, and the kidneys were removed and perfused via the renal artery, with a solution consisting of nonbicarbonate Ringer (containing NaCl, 130 mM; KCl, 5 mM; CaCl₂, 1 mM; MgSO₄, 1 mM; NaH₂PO₄, 1 mM; Tris-Hepes, 10 mM; pH 7.4) diluted 1:1 with Joklik's medium containing 10% fetal bovine serum (Joklik's). The kidneys were perfused with iced medium until the effluent was clear of blood, and the inner medullae were excised and placed in Joklik's me-

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1. Abbreviations used in this paper: ANP, atrial natriuretic peptide; GFR, glomerular filtration rate; HOWI, head-out water immersion; IBMX, isobutyl methyl xanthine; IMCD, inner medullary collecting duct; QO₂, oxygen consumption.

dium. Medullas were minced and subjected to dispersion in 0.2% collagenase for 90 min at 37°C in a shaking water bath. During the incubation, the suspension was agitated and aspirated intermittently, using a Pasteur pipette to break up clumps of cells, tubule fragments, and connective tissue. The resulting suspension of inner medullary cells was layered on 16% Ficoll in nonbicarbonate Ringer's and centrifuged at 4°C at 2,300 g for 45 min. IMCD cells were harvested from the interface between the Joklik's medium and the Ficoll. Cells were used within 2 h of preparation, and were kept on ice until study. Homogeneity of the preparation was assessed by phase contrast microscopy. There were no smooth muscle or endothelial cells visible in the preparations. Cell viability was greater than 90%, using trypan blue exclusion. IMCD cells prepared in this manner exhibit high glycolytic capacity (13, 17), inhibition of Na⁺ transport with amiloride (14, 16), increased cAMP accumulation in response to arginine vasopressin (AVP) (13), and increased cGMP accumulation in response to ANP₍₉₉₋₁₂₆₎ (13), all properties attributed to this segment in vivo.

Oxygen consumption (QO₂). QO₂ was measured as described previously (18, 19), in sealed continuously stirred water-jacketed 400- μ l glass chambers at 37°C. Nonbicarbonate Ringer's medium contained 10 mM pyruvate and 2 mM acetate, pH 7.4. After a control period to establish a stable baseline, additions of agents were made in volumes of < 1% of the volume of the chamber to eliminate potential dilutional problems; addition of DMSO or incubation buffer did not alter QO₂. The rate of QO₂ was calculated from the slope of the recorded line by measuring its angle against the baseline and calculating its tangent. When the measurement of QO₂ was ended, an aliquot of the cell suspension was removed and its wet weight was determined as described (18, 19). Results are expressed as millimoles of O₂ consumed per gram wet weight.

⁸⁶Rb⁺ uptakes. ⁸⁶Rb⁺ uptakes were performed in cells preincubated for 60 min in K⁺-free (substitution of NaCl for KCl, on a mole for mole basis) nonbicarbonate Ringer's medium with 5 mM glucose as sole substrate (17, 18). At the end of the incubation, cells were treated for 5 min with peptide or vehicle. Uptakes were then initiated by the addition of ⁸⁶Rb⁺ (0.7 μ Ci) in KCl (final K⁺ concentration, 5 mM), and at the indicated times, 60- μ l aliquots of the cell suspension were placed in 600 μ l of a stop solution, and the cells were pelleted through oil as described (17, 18, 20). Specific activity of ⁸⁶Rb⁺ was determined by dispersing the pellet in 6% perchloric acid overnight, neutralizing the pH, and counting in a liquid scintillation counter in Aquasol. Uptakes were performed with and without the indicated peptides in the presence and absence of ouabain. The uptake in the presence of ouabain (< 30% of total uptake) was subtracted from that obtained in the absence of ouabain to give the ouabain-sensitive uptake; at no time point did the peptides alter the ouabain-insensitive uptake.

PGE₂ assay. IMCD cells were preincubated for 30 min at 37°C in nonbicarbonate Ringer with 5 mM glucose. The suspension was sampled to provide basal PGE₂ content, and either peptide or vehicle was added. After 5 min incubation at 37°C, the reaction was stopped. PGE₂ was extracted from all samples by methanol-ethanol extraction. 2 vol of ice-cold ethanol was added to each sample, and the mixture allowed to stand at 4°C for 60 min. The mixture was centrifuged at 2,000 g for 10 min, and the supernatant was retained. The pellet was resuspended in two volumes of methanol, allowed to stand, and centrifuged as described above. The supernatants were pooled, dried under N₂, and assayed for PGE₂, using a commercial radioimmunoassay kit.

cGMP accumulation studies. IMCD cells were incubated at 37°C for 10 min before a further preincubation with 1 mM isobutyl methyl xanthine (IBMX) for 2 min in preparation for measurement of cGMP accumulation. cGMP accumulation was measured in the pre-equilibrated cells over a 2-min incubation in the presence or absence of added peptide. After 2 min, the cells were precipitated with 12% TCA. The TCA was extracted four times with 10 vol of water-saturated ether before assay of cGMP (13, 20).

Materials. ⁸⁶Rb⁺, assay kits for determination of PGE₂, and Aquasol were obtained from New England Nuclear, Boston, MA. Dioc-

tyllphthalate and silicon oil were obtained from Aldrich Chem. Co., Milwaukee, WI. Collagenase was obtained from Worthington Biochemical Corp., Freehold, NJ. Joklik's Minimal Essential Medium was obtained from Gibco Laboratories, Grand Island, NY, and Ficoll was obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Protein assay kits were obtained from Bio-Rad Chemical Division, Richmond, CA. cGMP radioimmunoassay kits were purchased from Biomed. Technol., Stoughton, MA. Natriuretic peptides (ANP₍₃₁₋₆₇₎ and ANP₍₉₉₋₁₂₆₎) were obtained from Peninsula Laboratories Inc., Belmont, CA. All other reagents were obtained from Sigma Chemical Co., St. Louis, MO, and were of analytical grade.

Statistics. In all figures, a single *n* represents the mean of determinations performed on a single cell preparation. Unless specified otherwise, experimental results are given for the mean \pm SE for four to eight separate cell preparations. Where appropriate, results of different groups are compared using unpaired or paired *t* tests as indicated; *P* < 0.05 was deemed significant.

Results

To determine whether ANP₍₃₁₋₆₇₎ altered Na⁺ transport in IMCD cells, the effect of this peptide on QO₂ was examined. Basal rates of QO₂ were 0.31 \pm 0.04 μ mol/min per g wet wt (*n* = 11), a value similar to previous results (13, 16–18). As shown in the upper panel of Fig. 1, the addition of vehicle to the incubated cells caused no discernible change in the rate of oxygen consumption, whereas addition of 10⁻⁸ M ANP₍₃₁₋₆₇₎ reduced QO₂ on average by 26%. As shown in the lower panel of Fig. 1, inhibition of QO₂ was a function of increasing ANP₍₃₁₋₆₇₎ concentration, and this inhibitory effect was maximal at about 100 pM. Half-maximal inhibition of QO₂ occurred at \sim 10 pM, a value similar to that at which this peptide is thought to circulate in human plasma (9, 10).

Inhibition of QO₂ may be caused by a reduction in Na⁺ transport, reducing the demands of the Na⁺-K⁺-ATPase for metabolic energy, or by direct inhibition of mitochondrial O₂ consumption. To determine whether the effect on QO₂ was due to inhibition of Na⁺ transport, the effect of ANP₍₃₁₋₆₇₎ on QO₂ was measured in the presence or absence of either the

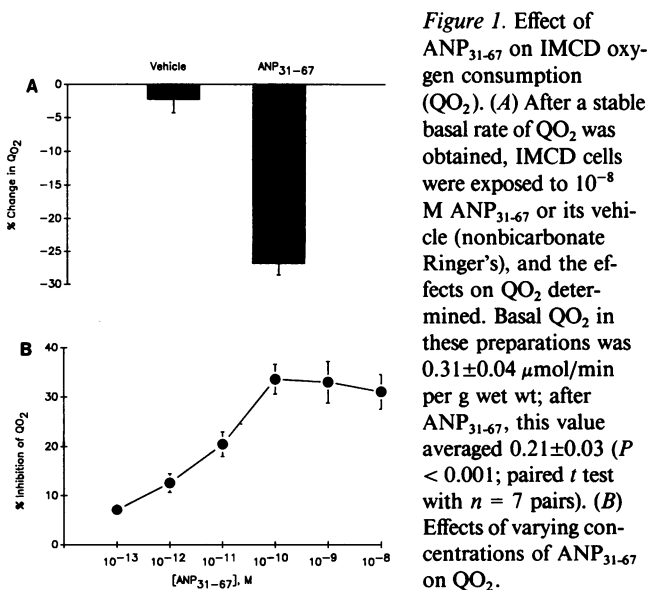


Figure 1. Effect of ANP₃₁₋₆₇ on IMCD oxygen consumption (QO₂). (A) After a stable basal rate of QO₂ was obtained, IMCD cells were exposed to 10⁻⁸ M ANP₃₁₋₆₇ or its vehicle (nonbicarbonate Ringer's), and the effects on QO₂ determined. Basal QO₂ in these preparations was 0.31 \pm 0.04 μ mol/min per g wet wt; after ANP₃₁₋₆₇, this value averaged 0.21 \pm 0.03 (*P* < 0.001; paired *t* test with *n* = 7 pairs). (B) Effects of varying concentrations of ANP₃₁₋₆₇ on QO₂.

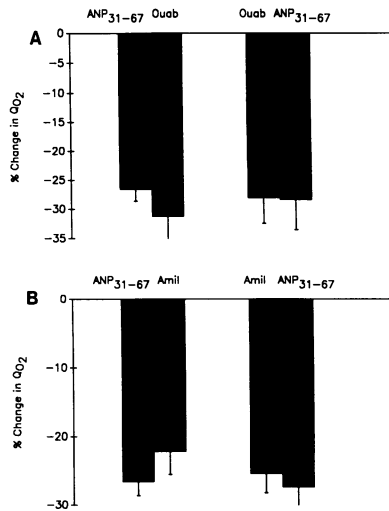


Figure 2. Interaction of ANP₃₁₋₆₇ inhibition of QO_2 with ouabain (A) and amiloride (B). After a stable baseline was obtained, cells were exposed to the agent indicated, followed by the second agent in the continued presence of the first agent.

Na⁺-K⁺-ATPase inhibitor ouabain, or the epithelial Na⁺ channel inhibitor amiloride. As shown in Fig. 2 A, the effect of addition of ouabain and ANP₍₃₁₋₆₇₎ was nonadditive, regardless of the order of additions. Thus, once the Na⁺-K⁺-ATPase was inhibited maximally by ouabain, the inhibitory effect of ANP₍₃₁₋₆₇₎ was no longer seen. Fig. 2 B demonstrates a similar set of experiments using the Na⁺ channel inhibitor amiloride. Amiloride, by inhibiting Na⁺ entry through the apical Na⁺ channel, reduces Na⁺-K⁺-ATPase activity and the consumption of metabolic energy by the pump. QO_2 inhibition by ANP₍₃₁₋₆₇₎ is not enhanced by amiloride, irrespective of the order of additions, whereas previous studies have shown that agents which directly reduce oxidative phosphorylation in IMCD cells, such as cyanide or glucose, give additive inhibition of QO_2 with ouabain or amiloride (16, 17). In additional studies, ANP₃₁₋₆₇ did not alter the rate of oxygen consumption obtained when mitochondrial oxygen metabolism was uncoupled with the mitochondrial poison, FCCP. These results indicate that ANP₍₃₁₋₆₇₎ reduces QO_2 not by direct inhibition of mitochondrial O₂ consumption, but by reducing the demand for metabolic energy of the Na⁺-K⁺-ATPase.

A reduction of transcellular Na⁺ transport could result from inhibition of Na⁺ entry via Na⁺ channels, or an inhibition of Na⁺-K⁺-ATPase itself. To determine which component of the Na⁺ transporting mechanism was primarily inhibited by ANP₍₃₁₋₆₇₎, we studied the effect of ANP₍₃₁₋₆₇₎ on cells that were permeabilized to Na⁺ with amphotericin B (10 μg/ml). Shown in Fig. 3 is the effect on QO_2 of amphotericin alone, or in combination with ANP₍₃₁₋₆₇₎ (B) or ANP₍₉₉₋₁₂₆₎ (A). Amphotericin alone caused a 25–35% stimulation of QO_2 by allowing the entry of excess Na⁺ into the cell and thus accelerating the activity of the Na⁺-K⁺-ATPase to V_{max} . In Fig. 3 B is shown the ANP₍₃₁₋₆₇₎ inhibition of QO_2 averaging 26%. Although this inhibition is reversed by the subsequent addition of amphotericin, the overall result of this combination is a net stimulation of QO_2 of only 7%. Thus, the inhibitory effect of the peptide is seen in its ability to attenuate the stimulation usually seen with amphotericin. If the order of additions is reversed, amphotericin increases QO_2 by 35% as expected, and the subsequent addition of ANP₍₃₁₋₆₇₎ inhibits 66% of this stimulation. Thus, from these data we conclude that ANP₍₃₁₋₆₇₎ has an inhibitory effect

on the rate of pumping by the Na⁺-K⁺-ATPase. By way of comparison, Fig. 3 A demonstrates the interaction of ANP₍₉₉₋₁₂₆₎ and amphotericin. Although ANP₍₉₉₋₁₂₆₎ inhibits QO_2 to a degree similar to that of ANP₍₃₁₋₆₇₎, subsequent addition of amphotericin not only reverses this inhibition but also stimulates QO_2 by ~ 25%, as would be expected from amphotericin alone, suggesting that the inhibition by ANP₍₉₉₋₁₂₆₎ was not due to direct inhibition of the Na⁺-K⁺-ATPase. Furthermore, prior stimulation of Na⁺-K⁺-ATPase with amphotericin blocks the action of ANP₍₉₉₋₁₂₆₎. Thus, ANP₍₃₁₋₆₇₎ appears to inhibit the Na⁺-K⁺-ATPase, an effect entirely different than the Na⁺ channel blocking effect of ANP₍₉₉₋₁₂₆₎.

To determine more directly whether ANP₍₃₁₋₆₇₎ inhibits Na⁺-K⁺-ATPase, rates of ouabain-sensitive ⁸⁶Rb⁺ uptake into IMCD cells were examined in the presence or absence of ANP₍₃₁₋₆₇₎. To insure that any effects measured were not due to regulatory effects of Na⁺ entry on the pump, Na⁺-K⁺-ATPase activity was maximally accelerated by preincubation of the cells in K⁺-free Ringer's followed by addition of isotope in the

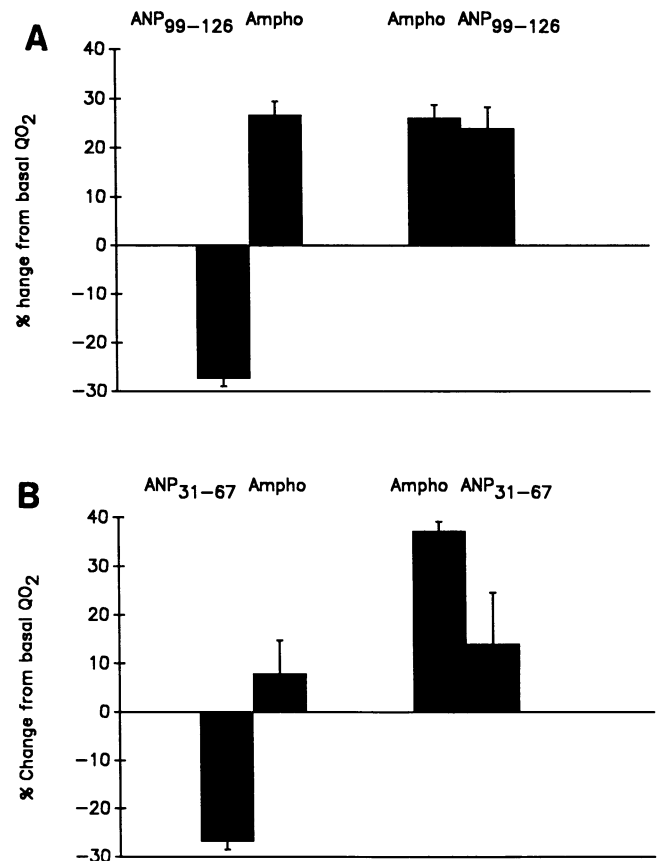


Figure 3. Interaction of ANP₃₁₋₆₇ or ANP₉₉₋₁₂₆ inhibition of QO_2 with amphotericin B. (A) On the left, ANP₉₉₋₁₂₆ was added, followed by amphotericin B in the continued presence of ANP₉₉₋₁₂₆; on the right, the order of additions was reversed. (B) On the left, ANP₃₁₋₆₇ was added, followed by amphotericin B in the continued presence of ANP₃₁₋₆₇; on the right, the order of additions was reversed. QO_2 after addition of ouabain averaged 0.38 ± 0.06 μmol/min per g wet wt; if amphotericin was added after exposure of the cells to ANP₃₁₋₆₇, QO_2 averaged 0.23 ± 0.05 ($P < 0.005$; paired *t* test comparing amphotericin first or after ANP₃₁₋₆₇ in five paired experiments). Data for A were taken from reference 16.

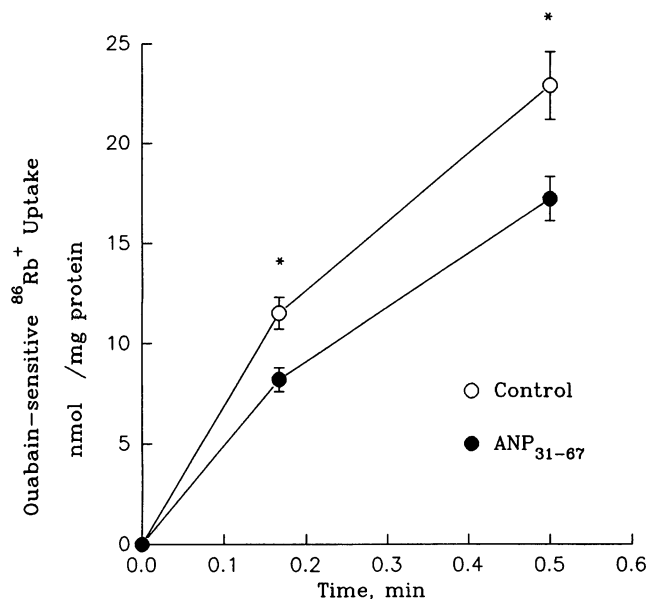


Figure 4. Effect of ANP₃₁₋₆₇ on ouabain-sensitive ⁸⁶Rb⁺ uptake. To deplete intracellular K⁺ and increase intracellular Na⁺, cells were preincubated 60 min in K⁺-free nonbicarbonate Ringer at 37°C. Uptakes were initiated by abrupt addition of ⁸⁶Rb⁺ and sufficient K⁺ to achieve a final concentration of 5 mM. Cells were exposed to 10⁻⁸ M ANP₃₁₋₆₇ or vehicle (K⁺-free Ringer) for 5 min before and during the entire period of the uptake measurement. Each experiment was performed in the presence or absence of 1 mM ouabain, and the uptake in the presence of ouabain was subtracted from that in the absence of ouabain. Data are expressed as ouabain-sensitive uptakes in nmol/mg protein. In unpaired *t* tests, *P* < 0.005 at 10 s and *P* < 0.01 at 30 s (*n* = 10).

presence of potassium (17, 18). As shown in Fig. 4, ANP₍₃₁₋₆₇₎ (open circles) significantly reduced ⁸⁶Rb⁺ uptake, as compared with cells incubated with vehicle (filled circles). ANP₍₃₁₋₆₇₎ did not alter 30-min equilibrium uptakes (data not shown). To compare initial uptake rates, uptakes were fitted as well to a closed two-compartment model (see reference 14): $\ln(1 - U_t/U_\infty) = kt$ where U_t equals the uptake at time, t , U_∞ equals the uptake at equilibrium, and K equals the initial rate constant. Correlation coefficients for these fits averaged 0.992 ± 0.003 , indicating that the data fit this model well. Rate constants for vehicle-treated cells averaged 0.13 ± 0.02 , and the average for cells treated with ANP₃₁₋₆₇ averaged 0.08 ± 0.01 (*P* < 0.005; paired *t* test with *n* = 10). Thus, ANP₃₁₋₆₇ markedly reduced basal Na/K-ATPase activity, as indicated by QO₂ under basal conditions (see Fig. 2), and partially inhibited the pump under V_{max} conditions (see Figs. 3 and 4).

Because we have previously observed a similar effect on Na⁺-K⁺-ATPase activity by PGE₂ and peptides (e.g., endothelin and IL-1) which stimulate PGE₂ accumulation (17, 18a), the role of PGE₂ in ANP₍₃₁₋₆₇₎ action was examined. If PGE₂ mediates inhibition of Na⁺-K⁺-ATPase by ANP₍₃₁₋₆₇₎, then we would predict that: (a) ANP₍₃₁₋₆₇₎ and PGE₂ should give equivalent nonadditive inhibition of Na⁺-K⁺-ATPase activity; (b) inhibition of cyclooxygenase should blunt the inhibitory effect of ANP₍₃₁₋₆₇₎; and (c) ANP₍₃₁₋₆₇₎ should stimulate PGE₂ production under conditions similar to those yielding inhibition of Na⁺-K⁺-ATPase. As shown in Fig. 5, ouabain-sensitive ⁸⁶Rb⁺

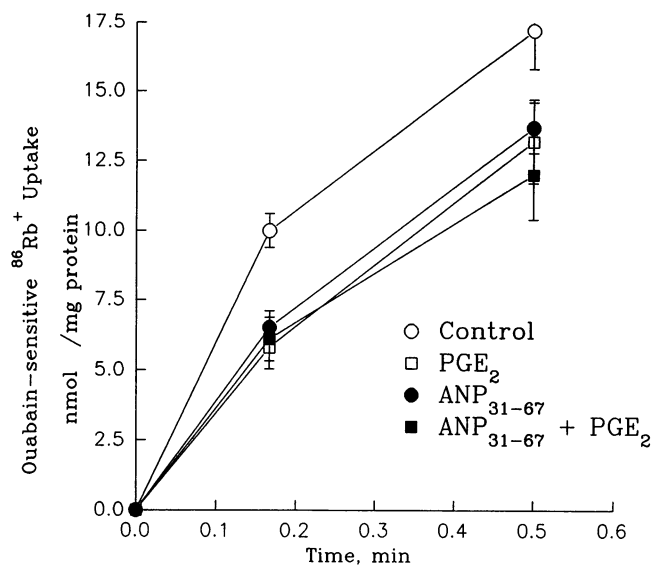


Figure 5. Effect of ANP₃₁₋₆₇, PGE₂, or both agents together on ouabain-sensitive ⁸⁶Rb⁺ uptake. Uptakes were performed as described in Fig. 4. Where indicated, PGE₂ was present at 10⁻⁶ M.

uptake was measured under four different conditions; control cells (open circles), cells exposed to PGE₂ (closed squares), or ANP₍₃₁₋₆₇₎ (closed circles), or both PGE₂ and ANP₍₃₁₋₆₇₎ (open squares). The inhibition of ouabain-sensitive ⁸⁶Rb⁺ uptake, compared with control, was the same at both 10 s and 30 s with PGE₂ alone, with ANP₍₃₁₋₆₇₎ alone, or with both agents together.

In Fig. 6, the effect of ibuprofen, a cyclooxygenase inhibitor, on ANP₍₃₁₋₆₇₎ inhibition of ouabain-sensitive ⁸⁶Rb⁺ was

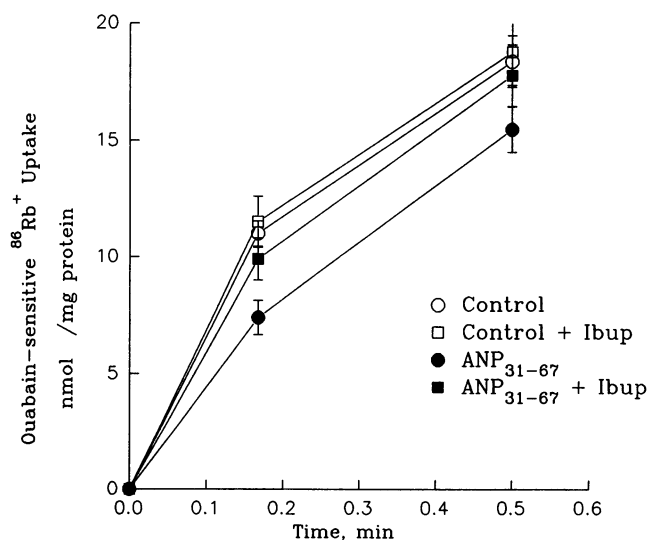


Figure 6. Effect of ibuprofen on ANP₃₁₋₆₇ inhibition of ouabain-sensitive ⁸⁶Rb⁺ uptake. Uptakes were performed as described in Fig. 4. Cells were preincubated in the ethanol vehicle for ibuprofen or 10⁻⁴ M ibuprofen for 10 min, exposed to PGE₂ or its vehicle (ethanol) for 5 min, and the uptakes were performed. Using a paired *t* test to compare the absolute rates of uptake with ANP₃₁₋₆₇ in the presence or absence of ibuprofen, *P* < 0.001 at 10 s and *P* < 0.005 at 30 s (*n* = 6 pairs).

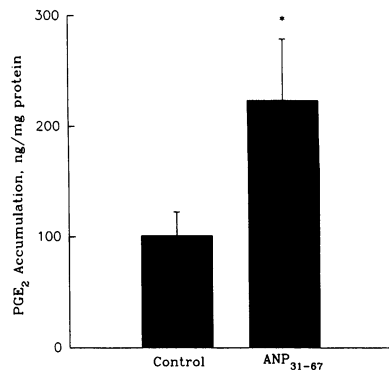


Figure 7. Effect of ANP₃₁₋₆₇ on IMCD PGE₂ production. Cells were exposed to 10⁻⁸ M ANP₃₁₋₆₇ or its vehicle (nonbicarbonate Ringer's) for 5 min and PGE₂ production determined. Results are expressed as nanogram accumulated per milligram cell protein. **P* < 0.05 compared with control.

measured. Cells were exposed to ibuprofen or its vehicle (ethanol), and then treated with ANP₃₁₋₆₇ of buffer before initiation of the uptake measurement. Pretreatment with ibuprofen markedly attenuated the inhibitory effect of ANP₃₁₋₆₇ on Na⁺-K⁺-ATPase activity.

Studies shown in Fig. 7 examine the effects of ANP₍₃₁₋₆₇₎ on PGE₂ production by IMCD cells. Control IMCD cells were incubated with vehicle alone, and produced 101.4 ± 21.6 ng PGE₂/mg protein. Incubation with ANP₍₃₁₋₆₇₎, however, stimulated the cells to double their production of PGE₂ (223.6 ± 55.5 ng/mg protein; *P* < 0.05 compared with control). Thus ANP₍₃₁₋₆₇₎ stimulates the production of PGE₂ in IMCD cells.

To determine whether ANP₍₃₁₋₆₇₎ might stimulate cGMP accumulation (21), IMCD cells were incubated with either ANP₍₉₉₋₁₂₆₎ or ANP₍₃₁₋₆₇₎ in the presence of IBMX, and cGMP generation was measured. Since the inhibition of QO₂ effected by ANP₃₁₋₆₇ is first apparent at 1–2 min after addition of the peptide, cGMP levels were determined after 2 min of exposure to the peptide. It was anticipated that if cGMP mediated the actions of ANP₃₁₋₆₇, the levels of cGMP would rise before, or simultaneously with, the observed inhibition of QO₂. As shown in Fig. 8, ANP₍₉₉₋₁₂₆₎ stimulated cGMP accumulation up to 20-fold at maximal (1 μM) concentrations, as observed previously (13), whereas ANP₍₃₁₋₆₇₎ had no discernable stimulatory effect even at maximal concentrations.

Discussion

The original descriptions of the natriuretic effect of an extract of rat atria in bioassay rats demonstrated for the first time that the heart elaborated a substance that could contribute to Na⁺ homeostasis (1). The structure of this atrial natriuretic factor was rapidly elucidated (4). The protein is synthesized as a prepeptide of 151 amino acids which, after removal of the signal peptide, is stored as a 126 amino acid pro-ANP (ANP₁₋₁₂₆). After release of ANP₁₋₁₂₆ from atrial cells, the final 28 amino acids from the carboxy terminal are cleaved off, forming ANP₉₉₋₁₂₆ (4–6). Because this carboxy-terminal product was readily detectable and exhibited nearly all of the actions of atrial extracts, virtually all subsequent studies focused on the actions of ANP₍₉₉₋₁₂₆₎. However, early studies also showed that intact ANP₍₁₋₁₂₆₎ exhibits biological activity (5). More recent evidence suggests that fragments of ANP₍₁₋₉₈₎ also circulate in humans, are regulated by appropriate changes in volume status, are elevated in disease states, and have a natriuretic effect in the kidney.

Studies examining the potential role of portions of the ANP₁₋₉₈ peptide used selective antisera raised against synthetic fragments of the ANP₍₁₋₉₈₎ peptide to identify circulating forms of ANP. Itoh et al. (7) demonstrated the secretion of a 10-kD fragment with the 3-kD ANP₍₉₉₋₁₂₆₎, while Melegros et al. suggested that the circulating amino portion of ANP₁₋₉₈ encompassed the amino acids 1–67 (cardiodilatin) (22). Winters et al. demonstrated that antibodies that selectively recognize ANP₃₁₋₆₇ and not other portions of ANP₁₋₁₂₆ detected a circulating peptide in human plasma (8, 9). This circulating peptide coeluted with authentic synthetic ANP₃₁₋₆₇ on reverse phase HPLC, and was shown by gel filtration to have a molecular mass of 4,000, similar to that of authentic ANP₃₁₋₆₇ (9). They further demonstrated that, like ANP₉₉₋₁₂₆, this peptide circulates at increased concentrations after HOWI, and in patients with congestive heart failure (8–10). Importantly, this peptide achieves higher plasma levels and exhibits a longer half-life than ANP₉₉₋₁₂₆ (8–10). Finally, Martin et al. demonstrated that injection of synthetic ANP₃₁₋₆₇ into rats stimulates natriuresis (11). The natriuresis was similar in magnitude to that seen with ANP₍₉₉₋₁₂₆₎, and occurred in the absence of a detectable change in the GFR.

Thus, ANP₃₁₋₆₇ or a nearly identical peptide circulates at levels that respond to changes in atrial pressure, is biologically active, and may play an important role in the regulation of salt balance in mammals. Furthermore, its prolonged half-life over that of ANP₍₉₉₋₁₂₆₎ suggests that the atrial natriuretic peptide system may respond in an acute fashion via ANP₍₉₉₋₁₂₆₎, and in a more prolonged fashion via the ANP₍₃₁₋₆₇₎ moiety.

Little is known of the mechanism by which ANP₍₃₁₋₆₇₎ exerts its renal actions. Our results show that unlike ANP₍₉₉₋₁₂₆₎, which inhibits Na⁺ entry through the amiloride-sensitive Na⁺ channel (14, 15), ANP₍₃₁₋₆₇₎ is a potent inhibitor of

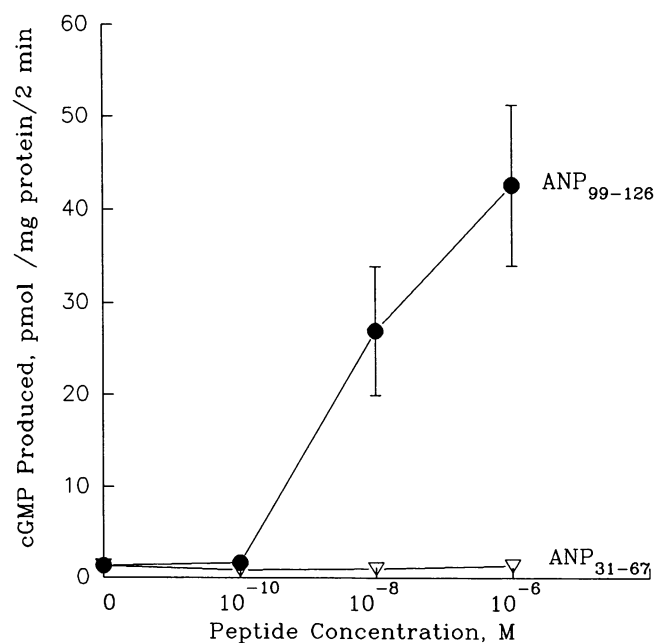


Figure 8. Effect of ANP₃₁₋₆₇ and ANP₉₉₋₁₂₆ on IMCD cGMP production. Cells were treated for 5 min at 37°C with the peptides as indicated in the presence of 10⁻⁴ M isobutylmethylxanthine (IBMX) before extraction and assay of cGMP accumulation.

Na⁺-K⁺-ATPase activity in IMCD cells. From the data presented in Figs. 1–4, it can be concluded that the peptide inhibits transcellular Na⁺ transport in IMCD cells at concentrations similar to those observed in vivo. Two approaches, each designed to isolate effects on Na⁺-K⁺-ATPase from effects on Na⁺ entry, demonstrated inhibition of the pump. In the studies of QO₂, amphotericin B was used to allow rapid Na⁺ entry into the cells, maximally stimulating the pump (17–19). The inhibitory effect of ANP₍₃₁₋₆₇₎, but not of ANP₍₉₉₋₁₂₆₎ in this setting strongly suggests that ANP₍₃₁₋₆₇₎ inhibits pump activity itself. This impression was confirmed by measurements of ouabain-sensitive ⁸⁶Rb⁺ uptake. The uptake measurements were performed in cells preincubated in K⁺-free medium. In the absence of K⁺, the pump is reversibly inhibited, leading to accumulation of intracellular Na⁺ and depletion of intracellular K⁺. The finding of inhibition of uptake under these conditions demonstrates that ANP₍₃₁₋₆₇₎ acts on the pump itself, and does not reduce pump activity by inhibiting Na⁺ entry pathways.

This mechanism of action distinguishes ANP₍₃₁₋₆₇₎ from ANP₍₉₉₋₁₂₆₎ because the binding of the latter to its receptor stimulates cGMP accumulation which in turn reduces Na⁺ entry via the amiloride-sensitive cation channel (12, 14, 15, 23, 24). The demonstration that ANP₍₃₁₋₆₇₎ did not stimulate cGMP accumulation, despite concurrent inhibition of phosphodiesterase by IBMX, again favors a direct inhibition of pump activity, and prompted a search for a different intracellular mediator.

PGE₂ stimulates natriuresis in the distal nephron (25, 26), and acute infusion of normal saline elicits an increase in renal prostaglandin excretion. Direct transport effects of PGE₂, including inhibition of net Na⁺ reabsorption, have been demonstrated in isolated cortical and medullary collecting tubules (25, 27). We have previously shown that PGE₂ inhibits Na⁺-K⁺-ATPase activity in IMCD cells (17). More recently, Strange has demonstrated that exposure of isolated perfused cortical collecting duct to basolateral PGE₂ leads to cell swelling, an effect strikingly similar to that obtained with basolateral ouabain (28). These results indicate that PGE₂ inhibits Na⁺-K⁺-ATPase in cortical and IMCDs.

In this regard, it is of interest that Epstein et al. have demonstrated that the natriuresis, after HOWI, is accompanied by an increase in PGE excretion (29); because of the enormous renal medullary PGE₂ synthetic capacity, excretion is thought to reflect de novo medullary PGE₂ synthesis. Prior treatment of subjects with the cyclooxygenase inhibitor, indomethacin, blunted both the natriuresis and PGE excretion, particularly in volume-depleted subjects. Thus, the natriuretic effect of HOWI, which is thought to involve increased venous return to the heart, atrial stretching, and release of atrial natriuretic peptides, is dependent at least in part on intact prostaglandin synthesis.

These considerations prompted us to examine the role of PGE₂ in the mediation of the ANP₍₃₁₋₆₇₎ response. Our data show that the inhibition of pump activity by this peptide is equivalent and nonadditive with PGE₂, and is markedly attenuated by pretreatment of the IMCD cells with ibuprofen. In addition, ANP₍₃₁₋₆₇₎ stimulates PGE₂ production in IMCD cells under conditions similar to those used to examine transcellular Na⁺ transport in these cells. These results provide strong evidence that PGE₂ mediates the inhibitory effect of ANP₍₃₁₋₆₇₎ on Na⁺-K⁺-ATPase activity. We have recently demonstrated a similar role for PGE₂ in mediating inhibition of IMCD Na⁺

transport by IL1 and endothelin (18, 18a); these peptides, like ANP₍₃₁₋₆₇₎, have also been shown to stimulate natriuresis in intact animals by predominantly tubule mechanisms. These results suggest a common mechanism by which renal medullary production of PGE₂ mediates the actions of several natriuretic peptides in vivo.

Thus, our data suggest that atrial peptides exert their natriuretic effects by more than one mechanism in the IMCD cell. The previously described mechanism of guanylate cyclase activation by ANP₍₉₉₋₁₂₆₎ may be important for the acute regulation of Na⁺ transport, while inhibition of IMCD Na⁺-K⁺-ATPase activity by ANP₍₃₁₋₆₇₎ may be important in more long-term regulation of Na⁺ balance.

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