Atrial Natriuretic Peptide$_{(31-67)}$ Inhibits Na^+ Transport in Rabbit Inner Medullary Collecting Duct Cells

Role of Prostaglandin E_2

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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 (QO_2); 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 QO_2. ANP$_{(31-67)}$ reduced the amphotericin-induced stimulation of QO_2 consistent with inhibition by this peptide of the Na^+-K^+-ATPase. In addition, ANP$_{(31-67)}$ reduced ouabain-sensitive $^{86}Rb^+$ uptake under V_{max} conditions. Several lines of evidence indicated that PGE_2, 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 PGE_2. (*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 simply 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; $CaCl_2$, 1 mM; $MgSO_4$, 1 mM; NaH_2PO_4, 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; QO_2, 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 (QO_2). QO_2 was measured as described previously (18, 19), in sealed continuously stirred water-jacketed 400-μl 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 QO_2. The rate of QO_2 was calculated from the slope of the recorded line by measuring its angle against the baseline and calculating its tangent. When the measurement of QO_2 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 O_2 consumed per gram wet weight.

$^{86}Rb^+$ uptakes. $^{86}Rb^+$ 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 $^{86}Rb^+$ (0.7 μCi) in KCl (final K^+ concentration, 5 mM), and at the indicated times, 60-μl aliquots of the cell suspension were placed in 600 μl of a stop solution, and the cells were pelleted through oil as described (17, 18, 20). Specific activity of $^{86}Rb^+$ 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.

PGE_2 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 PGE_2 content, and either peptide or vehicle was added. After 5 min incubation at 37°C, the reaction was stopped. PGE_2 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 N_2, and assayed for PGE_2, 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 preequilibrated 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. $^{86}Rb^+$, assay kits for determination of PGE_2, and Aquasol were obtained from New England Nuclear, Boston, MA. Dioctyllphthallate 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. Technols., 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±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 QO_2 was examined. Basal rates of QO_2 were 0.31±0.04 μmol/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 QO_2 on average by 26%. As shown in the lower panel of Fig. 1, inhibition of QO_2 was a function of increasing $ANP_{(31-67)}$ concentration, and this inhibitory effect was maximal at about 100 pM. Half-maximal inhibition of QO_2 occurred at ~ 10 pM, a value similar to that at which this peptide is thought to circulate in human plasma (9, 10).

Inhibition of QO_2 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 O_2 consumption. To determine whether the effect on QO_2 was due to inhibition of Na^+ transport, the effect of $ANP_{(31-67)}$ on QO_2 was measured in the presence or absence of either the

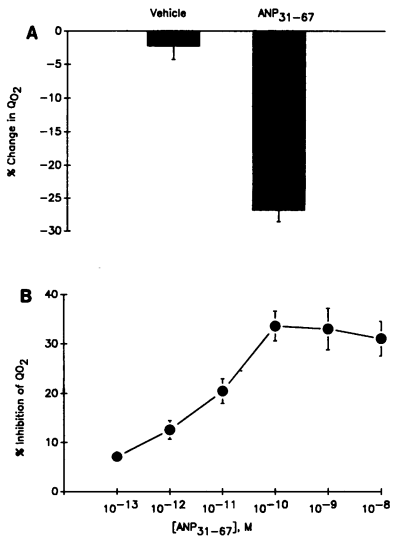

Figure 1. Effect of ANP_{31-67} on IMCD oxygen consumption (QO_2). (*A*) After a stable basal rate of QO_2 was obtained, IMCD cells were exposed to 10^{-8} M ANP_{31-67} or its vehicle (nonbicarbonate Ringer's), and the effects on QO_2 determined. Basal QO_2 in these preparations was 0.31±0.04 μmol/min per g wet wt; after ANP_{31-67}, this value averaged 0.21±0.03 ($P < 0.001$; paired *t* test with $n = 7$ pairs). (*B*) Effects of varying concentrations of ANP_{31-67} on QO_2.

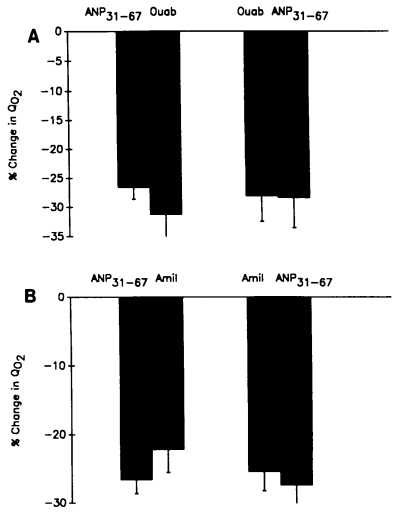

Figure 2. Interaction of ANP_{31-67} inhibition of QO_2 with ouabain (*A*) and amiloride (*B*). After a stable baseline was obtained, cells were exposed to the agent indicated, followed by the second agent in the continued presence of the first agent.

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, ANP_{31-67} 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 O_2 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 $^{86}Rb^+$ 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

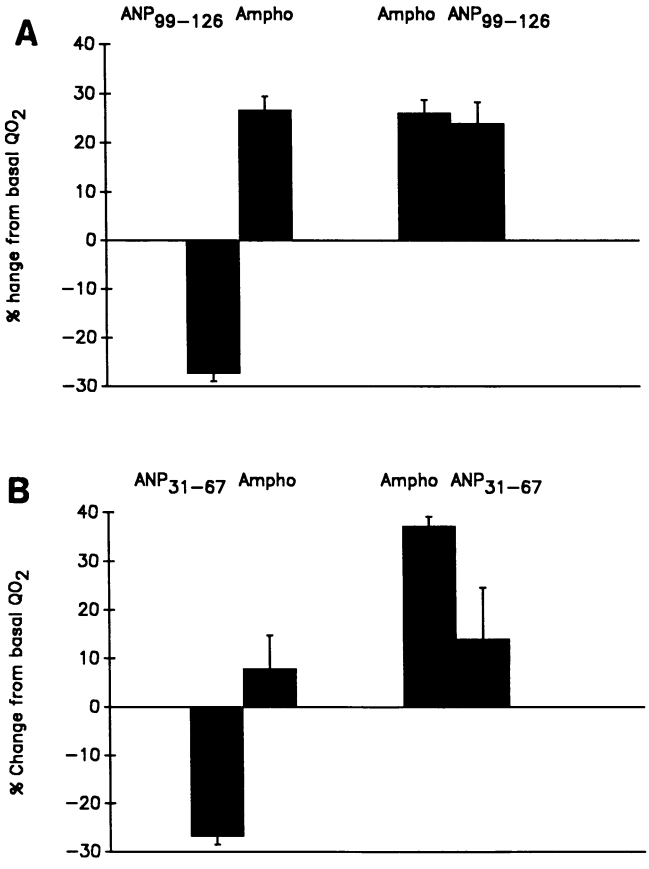

Figure 3. Interaction of ANP_{31-67} or ANP_{99-126} inhibition of QO_2 with amphotericin B. (*A*) On the left, ANP_{99-126} was added, followed by amphotericin B in the continued presence of ANP_{99-126}; on the right, the order of additions was reversed. (*B*) On the left, ANP_{31-67} was added, followed by amphotericin B in the continued presence of ANP_{31-67}; 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 ANP_{31-67}, QO_2 averaged 0.23±0.05 ($P < 0.005$; paired *t* test comparing amphotericin first or after ANP_{31-67} in five paired experiments). Data for *A* were taken from reference 16.

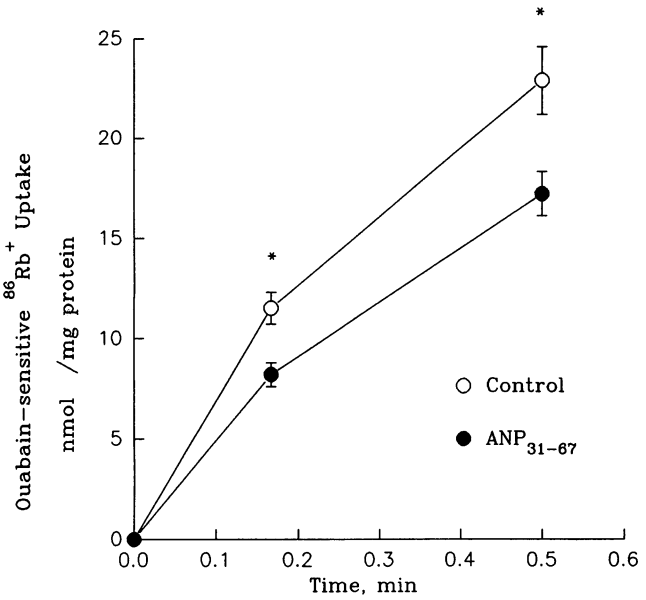

Figure 4. Effect of ANP_{31-67} on ouabain-sensitive $^{86}Rb^+$ uptake. To deplete intracellular K^+ and increase intracellular Na^+, cells were preincubated 60 min in K^+-free nonbicarbonate Ringer at 37°C. Uptakes were initiated by abrupt addition of $^{86}Rb^+$ and sufficient K^+ to achieve a final concentration of 5 mM. Cells were exposed to 10^{-8} M ANP_{31-67} 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 ($n = 10$).

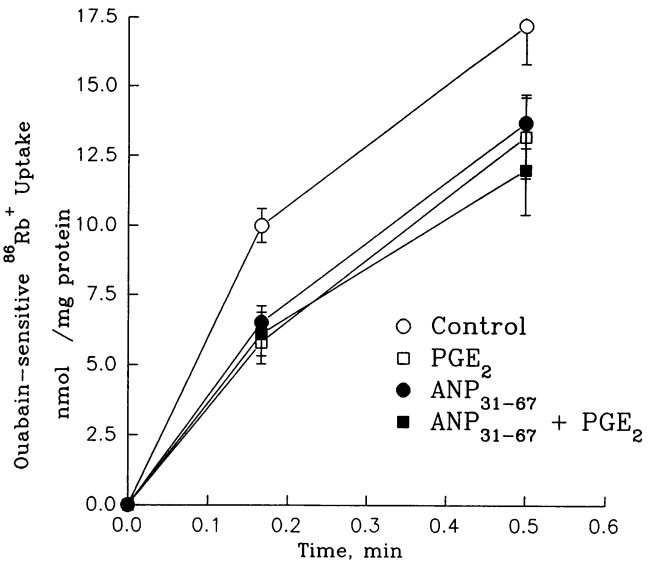

Figure 5. Effect of ANP_{31-67}, PGE_2, or both agents together on ouabain-sensitive $^{86}Rb^+$ uptake. Uptakes were performed as described in Fig. 4. Where indicated, PGE_2 was present at 10^{-6} M.

presence of potassium (17, 18). As shown in Fig. 4, $ANP_{(31-67)}$ (*open circles*) significantly reduced $^{86}Rb^+$ 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): $\text{Ln}(1 - U_t/U_\infty) = kt$ 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 ANP_{31-67} averaged 0.08±0.01 ($P < 0.005$; paired *t* test with $n = 10$). Thus, ANP_{31-67} markedly reduced basal Na/K-ATPase activity, as indicated by QO_2 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 PGE_2 and peptides (e.g., endothelin and IL-1) which stimulate PGE_2 accumulation (17, 18a), the role of PGE_2 in $ANP_{(31-67)}$ action was examined. If PGE_2 mediates inhibition of Na^+-K^+-ATPase by $ANP_{(31-67)}$, then we would predict that: (*a*) $ANP_{(31-67)}$ and PGE_2 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 PGE_2 production under conditions similar to those yielding inhibition of Na^+-K^+-ATPase. As shown in Fig. 5, ouabain-sensitive $^{86}Rb^+$ uptake was measured under four different conditions; control cells (*open circles*), cells exposed to PGE_2 (*closed squares*), or $ANP_{(31-67)}$ (*closed circles*), or both PGE_2 and $ANP_{(31-67)}$ (*open squares*). The inhibition of ouabain-sensitive $^{86}Rb^+$ uptake, compared with control, was the same at both 10 s and 30 s with PGE_2 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 $^{86}Rb^+$ was

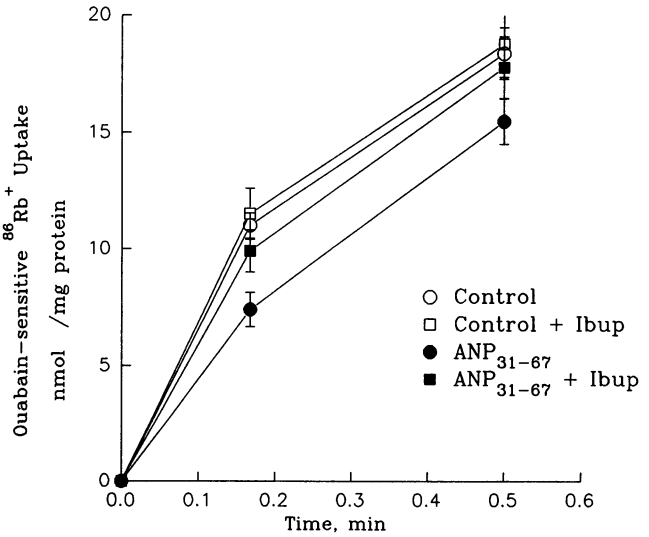

Figure 6. Effect of ibuprofen on ANP_{31-67} inhibition of ouabain-sensitive $^{86}Rb^+$ uptake. Uptakes were performed as described in Fig. 4. Cells were preincubated in the ethanol vehicle for ibuprofen or 10^{-4} M ibuprofen for 10 min, exposed to PGE_2 or its vehicle (ethanol) for 5 min, and the uptakes were performed. Using a paired *t* test to compare the absolute rates of uptake with ANP_{31-67} in the presence or absence of ibuprofen, $P < 0.001$ at 10 s and $P < 0.005$ at 30 s ($n = 6$ pairs).

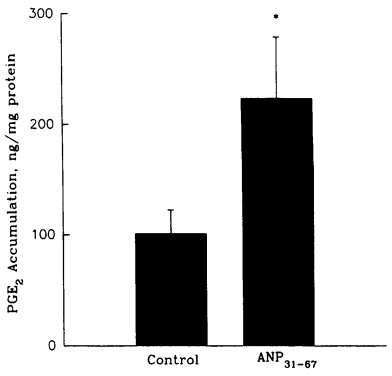

Figure 7. Effect of ANP_{31-67} on IMCD PGE_2 production. Cells were exposed to 10^{-8} M ANP_{31-67} or its vehicle (nonbicarbonate Ringer's) for 5 min and PGE_2 production determined. Results are expressed as nanogram accumulated per milligram cell protein. $*P < 0.05$ compared with control.

measured. Cells were exposed to ibuprofen or its vehicle (ethanol), and then treated with ANP_{31-67} of buffer before initiation of the uptake measurement. Pretreatment with ibuprofen markedly attenuated the inhibitory effect of ANP_{31-67} on Na^+-K^+-ATPase activity.

Studies shown in Fig. 7 examine the effects of $ANP_{(31-67)}$ on PGE_2 production by IMCD cells. Control IMCD cells were incubated with vehicle alone, and produced 101.4±21.6 ng PGE_2/mg protein. Incubation with $ANP_{(31-67)}$, however, stimulated the cells to double their production of PGE_2 (223.6 ±55.5 ng/mg protein; $P < 0.05$ compared with control). Thus $ANP_{(31-67)}$ stimulates the production of PGE_2 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 QO_2 effected by ANP_{31-67} 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 ANP_{31-67}, the levels of cGMP would rise before, or simultaneously with, the observed inhibition of QO_2. 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 prepropeptide of 151 amino acids which, after removal of the signal peptide, is stored as a 126 amino acid pro-ANP (ANP_{1-126}). After release of ANP_{1-126} from atrial cells, the final 28 amino acids from the carboxy terminal are cleaved off, forming ANP_{99-126} (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 ANP_{1-98} 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 Meleagros et al. suggested that the circulating amino portion of ANP_{1-98} encompassed the amino acids 1–67 (cardiodilatin) (22). Winters et al. demonstrated that antibodies that selectively recognize ANP_{31-67} and not other portions of ANP_{1-126} detected a circulating peptide in human plasma (8, 9). This circulating peptide coeluted with authentic synthetic ANP_{31-67} on reverse phase HPLC, and was shown by gel filtration to have a molecular mass of 4,000, similar to that of authentic ANP_{31-67} (9). They further demonstrated that, like ANP_{99-126}, 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 ANP_{99-126} (8–10). Finally, Martin et al. demonstrated that injection of synthetic ANP_{31-67} 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, ANP_{31-67} 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

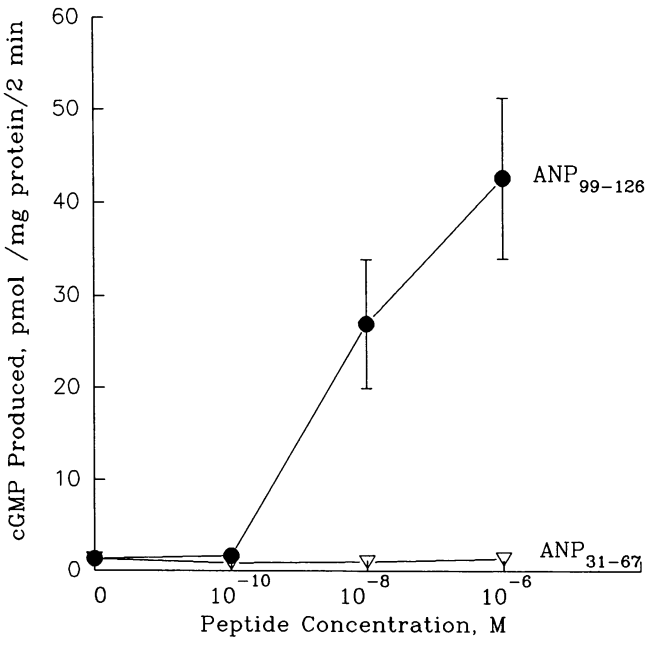

Figure 8. Effect of ANP_{31-67} and ANP_{99-126} on IMCD cGMP production. Cells were treated for 5 min at 37°C with the peptides as indicated in the presence of 10^{-4} M isobutylmethylxanthine (IBMX) before extraction and assay of cGMP accumulation.

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 QO_2, 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 $^{86}Rb^+$ 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.

PGE_2 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 PGE_2, including inhibition of net Na^+ reabsorption, have been demonstrated in isolated cortical and medullary collecting tubules (25, 27). We have previously shown that PGE_2 inhibits Na^+-K^+-ATPase activity in IMCD cells (17). More recently, Strange has demonstrated that exposure of isolated perfused cortical collecting duct to basolateral PGE_2 leads to cell swelling, an effect strikingly similar to that obtained with basolateral ouabain (28). These results indicate that PGE_2 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 PGE_2 synthetic capacity, excretion is thought to reflect de novo medullary PGE_2 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 PGE_2 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 PGE_2, and is markedly attenuated by pretreatment of the IMCD cells with ibuprofen. In addition, $ANP_{(31-67)}$ stimulates PGE_2 production in IMCD cells under conditions similar to those used to examine transcellular Na^+ transport in these cells. These results provide strong evidence that PGE_2 mediates the inhibitory effect of $ANP_{(31-67)}$ on Na^+-K^+-ATPase activity. We have recently demonstrated a similar role for PGE_2 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 PGE_2 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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