C-type natriuretic peptide inhibits ANP secretion and atrial dynamics in perfused atria: NPR-B-cGMP signaling

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The purpose of the present experiments was to define the role of C-type natriuretic peptide (CNP) in the regulation of atrial secretion of atrial natriuretic peptide (ANP) and atrial stroke volume. Experiments were performed in perfused beating and nonbeating quiescent atria, single atrial myocytes, and atrial membranes. CNP suppressed in a dose-related fashion the increase in atrial stroke volume and ANP secretion induced by atrial pacing. CNP caused a right shift in the positive relationships between changes in the secretion of ANP and atrial stroke volume or translocation of the extracellular fluid (ECF), which indicates the suppression of atrial myocyte release of ANP into the paracellular space. The effects of CNP on the secretion and contraction were mimicked by 8-bromoguanosine 3',5'-cyclic monophosphate (8-BrgGMP). CNP increased cGMP production in the perfused atria, and the effects of CNP on the secretion of ANP and atrial dynamics were accentuated by pretreatment with an inhibitor of cGMP phosphodiesterase, zaprinast. An inhibitor of the biological natriuretic peptide receptor (NPR), HS-142-1, attenuated the effects of CNP. The suppression of ANP secretion by CNP and 8-BrcGMP was abolished by a depletion of extracellular Ca\(^{2+}\) in nonbeating atria. Natriuretic peptides increased cGMP production in atrial membranes with a rank order of potency of CNP > BNP > ANP, and the effect was inhibited by HS-142-1. CNP and 8-BrcGMP increased intracellular Ca\(^{2+}\) concentration transients in single atrial myocytes, and mRNAs for CNP and NPR-B were expressed in the rabbit atrium. From these results we conclude that atrial ANP release and stroke volume are controlled by CNP via NPR-B-cGMP mediated signaling, which may in turn act via regulation of intracellular Ca\(^{2+}\).

atrial natriuretic peptide; guanosine 3',5'-cyclic monophosphate; guanylyl cyclase; calcium; natriuretic peptide receptor

SINCE THE DISCOVERY of atrial natriuretic peptide (ANP) in the cardiac atrium (6), brain natriuretic peptide (BNP) was found in the porcine brain (42) and then, later, in the cardiac atria (29). C-type natriuretic peptide (CNP), a third member of the natriuretic peptide family, was first isolated from the porcine brain (43), and, later, a high concentration was found in the anterior lobe of the pituitary gland in rats (25). Subsequently, it was shown that cultured vascular endothelial cells produce CNP (41, 44). Three types of natriuretic peptide receptors (NPRs) have been identified to date, namely, NPR-A, NPR-B, and NPR-C (3). From the binding characteristics and the rank order of potency of natriuretic peptides for the production of cGMP, it was suggested that ANP and BNP exert their effects mainly via NPR-A and CNP via NPR-B (24). CNP elicits natriuretic, diuretic, and hypotensive effects that are less potent than those of ANP or BNP (43). Because the plasma levels of CNP are very low, the presence of the peptide in tissues is considered to perform a paracrine/autocrine function as a local regulator. CNP has been shown to produce a variety of effects including an antiproliferative effect on vascular smooth muscle cells (10) and inhibition of luteinizing hormone secretion (37), ACTH-induced increase in aldosterone secretion (18), and basal secretion of arginine vasopressin from supraoptic nucleus neurons (47).

Recently, a regulatory function of CNP has been recognized in the heart, i.e., CNP-induced positive chronotropic (1, 2, 14) and inotropic (2, 14) effects in the dog heart. The natriuretic peptide family (11, 29, 46) and their specific receptors (2, 8, 33) have been demonstrated in the cardiac atria. However, intrinsic effects or cross-talk of the peptides, including regulation of ANP release via a short feedback loop, have not yet been clearly defined. Recently, Vesely et al. (45) suggested a negative feedback control of endogenous ANP for its own release via volume receptors in the heart of humans. Similarly, Leskinen et al. (26) reported that endogenous ANP modulates its own release via NPR-A in vivo experiments. In their experiments, HS-142-1 increased plasma levels of ANP and the NH\(_{2}\)-terminal fragment of pro-ANP, and ANP, but not CNP, blocked the volume expansion-stimulated increase in the NH\(_{2}\)-terminal fragment of pro-ANP. Nachshon et al. (31) further defined an autoregulatory mechanism of ANP secretion by atrial myocytes in a autocrine/paracrine manner. From the finding that NPR-A and NPR-B agonists (HS-142-1 and anantin, respectively) increase ANP release from cultured atrial myocytes, they suggested that ANP inhibits its own secretion via the NPR-A-cGMP pathway. Much work has focused on the regulatory mechanisms of ANP secretion. Mechanical...
stimulation has been considered to be the major stimulating factor for the regulation of the secretion of ANP (5, 7, 35). Receptor agonists including endothelin-1 have been shown to chemically stimulate the secretion of the cardiac hormones (35). However, the detailed roles for the stimuli are not clear.

From the fact that NPR-B and CNP are expressed in the cardiac atrium and CNP may possess paracrine regulatory functions in the local tissues, it is possible to postulate that there is a cross-regulation by CNP for mechanically stimulated ANP secretion in the atrium. The purpose of the present study was to define roles for CNP in the regulation of the secretion of ANP and atrial stroke volume in perfused atria. A schematic diagram of the study design and a path diagram of the responses are presented in Fig. 1.

METHODS

Beating Perfused Rabbit Atrial Preparation

New Zealand White rabbits were used. An isolated, perfused atrial preparation was prepared by the method described previously (5). Briefly, the hearts were removed, the left atrium was dissected, and a calibrated transparent atrial cannula containing two small catheters was inserted into the left atrium. The cannulated atrium was transferred to an organ chamber containing buffer solution at 36.5°C. The atrium was immediately perfused with HEPES buffer solution (1.25 ml/min). The composition of the buffer was as follows (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgCl2, 25 NaHCO3, 10.0 glucose, and 10.0 HEPES (with NaOH, pH 7.4) and 0.1% bovine serum albumin. Soon after setup of the perfused atrium, transmural electrical field stimulation with a luminal electrode was started at 1.3 Hz (duration 0.3–0.5 ms, voltage 20–30 V). The changes in atrial stroke volume were monitored by reading the lowest level of the water column in the calibrated atrial cannula during end diastole (5). Atrial pulse pressure was measured via a pressure transducer connected to the intra-atrial catheter and recorded on a physiograph. To estimate transendocardial extracellular fluid (ECF) translocation, transmural atrial clearance of [3H]inulin was measured as described previously (4, 5). Radioactivity in the atrial perfusate and pericardial buffer solution was measured, and the amount of ECF translocated through the atrial wall was calculated.

Nonbeating Quiescent Atrial Preparation

Only the left atria from rabbits and rats were used. Rabbit atrium was obtained as described previously. For the rat atria, female Sprague-Dawley rats (200–250 g) were killed by decapitation. An isolated, quiescent atrial preparation was prepared by the method described previously (19). Briefly, the cannulated atrium was transferred to an organ chamber containing buffer solution at 36.5°C, which was sealed with a watertight silicone rubber cap. The atrium was perfused with a buffer solution described previously (0.35 ml/min). The pericardial space of the organ chamber was sealed and connected to a calibrated microcapillary tube. Changes in atrial volume induced by atrial distension and removal of distension (atrial distension-reduction volume (DRV)) were monitored by measuring the length of the water column of the calibrated microtube.

Experimental Protocols

In beating atrial preparations, the atria were perfused for 60 min to stabilize ANP secretion. [3H]Inulin was introduced to the pericardial fluid 20 min before the start of the sample collection. The perfusate was collected at 2-min intervals at 4°C for analyses. Atrial pacing at 0.8, 1, 1.3, 1.6, and 2 Hz was performed consecutively for 2 min at each frequency and repetitive frequency change was done. Repetitive frequency changes were separated by 2 min of 0.8 Hz pacing. CNP-22 (Bachem) or 8-bromo-cGMP (8-BrcGMP) was introduced in the perfusate just after the third cycle. The effects of CNP or 8-BrcGMP were evaluated after two cycles (24 min) of the administration of the agent. Zaprinast, an inhibitor of phosphodiesterase type V, or HS-142-1, a microbial polysaccharide inhibitor of NPR-A and NPR-B, was introduced one cycle before the CNP administration (12 min before). In some experiments, atrial tissues were saved for measurement of cGMP production.

In nonbeating atrial preparations, the atria were perfused for 30 min to stabilize ANP secretion and to equilibrate extracellular space with [3H]inulin at a steady state. The perfusate was collected at 2-min intervals at 4°C. After two collection periods, atrial distension was induced by changing the elevation of the outflow catheter tip by 1, 2, 4, and 6 cmH2O above the atrium. Every 2-min period of atrial distension was followed by a reduction in atrial volume for 8 min produced by lowering the outflow catheter tip back to the basal level. To define the influence of Ca2+ on the effect of CNP, experiments were done in four groups of rat atria: atria perfused with regular buffer containing CNP or vehicle and atria perfused with Ca2+-free buffer containing CNP or vehicle.

Radioimmunoassay of ANP

Immunoreactive ANP in the perfusate was measured by a specific RIA, as described previously (4, 5). The secreted amount of immunoreactive ANP was expressed as nanograms of ANP per minute per gram of atrial tissue. The molar concentration of immunoreactive ANP released (4) was calu-
lated as follows

\[
\text{ANP released (µM)} = \frac{\text{immunoreactive ANP (in pg·min}^{-1}·\text{g}^{-1})}{\text{ECF translocated (in µl·min}^{-1}·\text{g}^{-1}·3.606)}}
\]

Most of the ANP secreted is processed ANP (4). CNP did not cross-react in the ANP assay.

Radioimmunoassay of cGMP

Production of cGMP was measured by equilibrated RIA (21). Briefly, standards or samples were incubated with antiserum for cGMP (Calbiochem-Novabiochem, San Diego, CA) and iodinated cGMP (guanosine 3′,5′-cyclic phosphoric acid, 2′-o-succinyl [125I]iodotyrosine methyl ester, NEN Life Science Products, Boston, MA) in a sodium acetate buffer (50 mM, pH 4.85) containing theophylline (8 mM). The bound form was separated from the free form by charcoal suspension. Nonspecific binding was <2.5%. The 50% intercept was at 0.39 ± 0.03 pmol/tube (n = 15). The intra- and interassay coefficients of variation were 6.7% (n = 12) and 8.6% (n = 9), respectively. For some experiments, we iodinated cGMP. To prepare iodinated cGMP we used 2′-o-monosuccinyl guanosine-3′,5′-cyclic monophosphate tyrosyl methyl ester (cGMP-TME, Sigma Chemical, St. Louis, MO) as recommended (40). cGMP-TME was iodinated by the chloramine T method. Iodinated cGMP-TME was purified by a QAE Sephadex A-25 column (Sigma Chemical). The specific activity (215 Ci/mmol) of the iodinated tracer was determined by an RIA technique (17). Nonspecific binding of iodinated tracer was <2.4%. The 50% intercept was at 0.74 ± 0.03 pmol/tube (n = 10). The intra- and interassay coefficients of variation were 4.2% (n = 15) and 7.1% (n = 8), respectively. Comparison of the values of cGMP obtained by RIA using two iodinated cGMP tracers (ours and NEN) shows a significant correlation (\( r = 0.9799 \pm 0.224, r = 0.995, P < 0.001, n = 8 \)). Results of the determinations were expressed as picomoles of cGMP generated per milligram of protein per minute.

Measurement of Particulate Guanylyl Cyclase Activity

Particulate guanylyl cyclase (GC) activity was measured by determination of cGMP generated in protein aliquots of the atrial membranes according to a method described previously (21). Briefly, aliquots of the protein suspension were incubated for 15 min at 37°C in Tris·HCl buffer (50 mM, pH 7.4) containing 1 mM GTP, 0.5 mM ATP, 15 mM creatine phosphate, 80 µg/ml creatine phosphokinase, 4 mM MgCl2 and CNP, BNP, or ANP. To test the specificity of GC-coupled NPRs, protein aliquots were also incubated in the incubation mixture with CNP plus HS-142-1. Incubations were stopped by adding 3 vols of ice-cold sodium acetate (50 mM, pH 5.8) and boiling for 5 min. Samples were then centrifuged at 10,000 g for 5 min at 4°C.

Preparation of Atrial Membranes for Measurement of Particulate GC Activity

The left atrium was obtained from adult (1.4–1.8 kg) New Zealand White rabbits. The atrial tissue was placed in 2 ml of ice-cold phosphate buffer (30 mM, pH 7.2) containing 120 mM NaCl and 1 mM phenanthroline, finely minced, and homogenized at 4°C by three 30-s bursts of maximal speed using a Tissue Tearor (Biospec Products, Racine, WI). The homogenate was centrifuged at 1,000 g for 10 min at 4°C, and the supernatant was recentrifuged at 40,000 g for 60 min at 4°C. The membrane pellet was washed three times with Tris·HCl buffer (50 mM, pH 7.4) containing 1 mM EDTA. The membranes were suspended with sonication in the above Tris·HCl buffer for the measurement of GC activity. Protein contents were determined by a biocinchonic acid assay kit (Sigma Chemical).

Preparation of Perfused Atrium for Measurement of cGMP Production

At the end of the perfusion experiments, the atria were separated from the atrial cannula, lightly blotted, quickly frozen in liquid nitrogen, and stored for 2 wk at −70°C. Atrial tissue was minced in 2 ml of ice-cold trichloroacetic acid (6%) solution and homogenized at 4°C by three 30-s bursts of maximal speed using the Tissue Tearor. Homogenates were centrifuged at 1,000 g for 10 min at 4°C, and the supernatant was transferred to a polypropylene tube, subjected to ether extraction three times, and then dried using a SpeedVac concentrator (Savant, Farmingdale, NY). Dried samples were resuspended with 200 µl of sodium acetate buffer.

Reverse Transcription and Polymerase Chain Reaction

To define the gene expression for CNP and NPRs, RNA extraction and RT-PCR were performed as described previously (20, 21). Briefly, total RNA was extracted from rabbit atria and rat pituitary gland and kidney using TRI reagents (MRC, Cincinnati, OH). One microgram of total RNA was sampled in twenty microliters of RT buffer and reverse transcribed at room temperature for 10 min and at 42°C for 30 min. The reaction was stopped by heat inactivation for 5 min at 99°C and then chilled on ice. Complementary DNA
products were amplified by PCR using primers. The sequences of the oligonucleotides for NPR-A and NPR-B were described previously (21). Gene specific primers were synthesized according to the published sequences (9, 23, 38). The sequences (5'–3') of the oligonucleotides and sizes of PCR products were: for NPR-A, sense: AAGAGCCTGATAATCGAGTACT, antisense: TTGCAGGCTGGCTCATTGTCA (451 bp); for NPR-B, sense: AAGGGGCGCATTGTTATCTGCAGGC, antisense: TTATCACAGGATGGGTCGTCCAAGTCA (692 bp); for NPR-C, sense: ATAGTGCGCTACATCCAGGGCAGT, antisense: TCCAAATATCAATCAATGACCTCCTGGTTACCTGC (573 bp); for ANP, sense: ATGGGCTCCTTCTCCATCCGAT, antisense: AGGGCCAGCGAGGCTTTGCT (363 bp); and for CNP, sense: CTCTCCAGCTGATCGCCTG, antisense: TAACATCCCAGCCGCCTCAT (374 bp). Because there is no report on the cDNA sequences of NPRs and CNP in the rabbit and the CNP gene and its expression is highly homologous between mammalian species, oligonucleotides were made on the basis of reports in rat (NPR-A, NPR-B, and ANP), porcine (CNP), and bovine (NPR-C) tissues. A hot-start PCR was used to increase the specificity of amplification. The temperature profile of amplification consisted of 30-s denaturation at 95°C, 1-min annealing at 60°C, and 2-min extension at 72°C for 40 cycles for ANP, NPR-A, and NPR-B and 1-min annealing at 65°C for 40 cycles for CNP and NPR-C. PCR products were separated in Fig. 3. Effect of CNP (3 × 10^{-7} M) on ANP secretion (A), extracellular fluid (ECF) translocation (B), atrial stroke volume (C), and ANP concentration (D) in beating rabbit atria (0.8, 1, 1.3, 1.6, 2.0 Hz) (○, CNP, n = 8; ●, control, n = 8). Relationships between ANP secretion and atrial stroke volume (E), ECF translocation and atrial stroke volume (F), and ANP secretion and ECF translocation (G) were examined. Values are means ± SE.
1.4% agarose gels, and bands were visualized by ethidium bromide staining. The specificity of the amplified sequences was confirmed by DNA sequencing.

Isolation of Atrial Myocytes

Isolation of atrial myocytes was performed as described previously (19). Briefly, Sprague-Dawley rats of either sex (200–250 g) were injected intraperitoneally with heparin (200 U/100 g body wt) and 30 min later anesthetized with pentobarbital sodium (50 mg/kg). The heart was perfused with oxygenated buffer solution containing 0.02 mM Ca²⁺ followed by a perfusion with solution containing 0.02 mM Ca²⁺. A collagenase buffer solution (110 U/ml, collagenase type 2, Worthington Biochemicals, Lakewood, NJ) was perfused for 1 min at a rate of 13.8 ml/min followed by a perfusion at a rate of 7.5 ml/min for 30 min with recirculation. Thereafter, the heart was perfused with oxygenated buffer solution (solution B) for 3 min to remove collagenase and the atrium was transferred to a culture dish in 3 ml of solution B. The composition of solution B was (in mM) 40 KCl, 50 L-glutamic acid, 20 taurine, 20 KH₂PO₄, 3 MgCl₂, 10 glucose, 0.5 EGTA, and 10 HEPES (pH 7.3). The atrium was dissected into small pieces, and single cells were isolated by gentle mechanical agitation for 2 min. Dispersed atrial cells were centrifuged and washed with solution A. Calcium-tolerant, rod-shaped cells with clear and regular cross-striations were used. Cells were used for the microscopy between 2 and 4 h after dispersion.

Measurement of Intracellular Calcium Ion Transients in Single Atrial Myocytes

Changes in intracellular Ca²⁺ concentration ([Ca²⁺]i) transients of single atrial myocytes were measured by fura 2 fluorescence digital imaging microscopy. Isolated cells were attached on coverslips coated with poly-L-lysine. The cells were incubated in buffer solution (solution A) containing 2 µM fura 2-AM (Molecular Probes, Eugene, OR) and 0.02% Pluronic F-127 (Molecular Probes) for 30 min at 35°C while being oxygenated (19). The cells were then washed with fresh oxygenated solution to remove extracellular dye. The cell-attached coverslip was transferred to a vessel and placed on the stage of an inverted fluorescence microscope (Axiovert 135, Karl Zeiss, Jena, Germany) attached to an Attofluor digital fluorescence microscopy system (Atto instruments, Rockville, MD). The cells were superfused with an oxygenated solution (0.5 ml/min) containing 0.4 mM Ca²⁺. The cells were imaged with excitation wavelength of 340 and 380 nm and emission wavelength of 480 nm. The fluorescence images were captured with an intensified charge-coupled device camera and analyzed with Attofluor image processing software. Changes in [Ca²⁺]i transients were presented as a ratio of fluorescence intensities obtained (F₃₄₀/F₃₈₀). Background subtraction was subtracted. Experiments were performed at room temperature (24–25°C).

Statistical Analysis

Significant difference from the control mean value after agent infusion was compared using two-way ANOVA for repeated measures (see Figs. 2–9). Student’s t-tests for paired (see Fig. 13) and unpaired (see Fig. 10) data were also applied. Differences in ratio increments (see Fig. 13B) were compared using Duncan’s multiple-range test. Significant differences from the control value after agent treatment in atrial membrane preparation were determined using one-way ANOVA followed by Bonferroni’s (see Fig. 11, A and B) and Dunnett’s (see Fig. 11C) multiple-comparison test. Statistical significance was defined as P < 0.05. The results are given as means ± SE.

RESULTS

Effects of CNP on Contraction and Secretion of Atrium

CNP inhibits atrial dynamics. At a fixed pacing frequency, atrial pulse pressure and stroke volume were stable during the experimental period (Fig. 2A, top). Repetitive frequency change in the range of 0.8–2 Hz resulted in stable and constant changes in atrial pulse pressure and stroke volume (Fig. 2A, bottom, and Fig. 2B). Increasing pacing frequency from 0.8 to 2 Hz increased atrial pulse pressure and stroke volume proportionally. CNP [10⁻⁸ (n = 9), 10⁻⁷ (n = 9), 3 x 10⁻⁷ (n = 8), and 10⁻⁶ M (n = 6)] suppressed the responses in a dose-dependent manner. The decrease in atrial stroke volume was significant at 3 x 10⁻⁷ and 10⁻⁶ M CNP (both P < 0.05, two-way ANOVA). Suppression by CNP (10⁻⁶ M) of the atrial dynamics appeared within 4 min of and continued steadily during the administration of the agent. No significant difference in atrial stroke

![Fig. 4. Effect of CNP on relationship between ANP secretion and ECF translocation. Values are means ± SE.](image-url)
volume was observed between periods corresponding to the control and experiment (P > 0.05, n = 7, time control).

CNP inhibits the release of ANP. An increase in pacing frequency resulted in an increase in secretion of ANP concomitantly with translocation of the ECF, which is coincident with an increase in atrial stroke volume (n = 8, Fig. 3, A–C). The concentration of ANP in perfusate in terms of the ECF translocation was 0.3–0.5 µM (Fig. 3D). Because the secretion of ANP is related to the atrial work in beating atria (Ref. 5 and present data), the response of ANP secretion was analyzed as a function of atrial stroke volume (Fig. 3E). Both secretion of ANP and translocation of the ECF were a function of atrial stroke volume (Fig. 3, E and F). At higher atrial stroke volumes, the incremental changes in the secretion of ANP and translocation of the ECF showed a peak and fall (Fig. 3, A and B). The change in ANP secretion was well correlated with translocation of the ECF (Fig. 3G).

CNP (3 × 10^{-7} M) suppressed the secretion of ANP (n = 8). The concentration of ANP in perfusate in terms of the ECF translocation was significantly decreased by CNP (P < 0.05, Fig. 3D). Translocation of the ECF in
response to atrial pacing was not changed by CNP (Fig. 3B), but the relationship between translocation of the ECF and atrial stroke volume was changed (P < 0.05, Fig. 3F). CNP shifted relationships between the secretion of ANP and atrial stroke volume or translocation of the ECF downward and rightward (P < 0.01 and P < 0.05, respectively; Fig. 3, E and G). This means that CNP suppresses myocytic release of ANP. This is related to the decrease in the concentration of ANP shown in Fig. 3D. A significant suppression of ANP secretion by CNP (P < 0.01, Fig. 3A) was observed and was dose dependent (Fig. 4). As shown in Fig. 4, CNP shifted the relationship between secretion of ANP and translocation of the ECF rightward in a dose-dependent manner. The changes in response to 3 × 10⁻⁷ (n = 8) and 10⁻⁶ M (n = 6) CNP were significant (both P < 0.05).

Changes in secretion of ANP and translocation of the ECF in response to repetitive changes in pacing frequency were constant and stable as shown by the time control of Fig. 4. The responses of the parameters were reproducible during the periods corresponding to the control and experimental observations of CNP (differences between periods were not significant; n = 7).

**Cellular Mechanisms Responsible for Effect of CNP**

Effect of CNP on contraction and secretion of atrium was mimicked by 8-BrcGMP. Because the generation of cGMP is generally accepted as a signal transduction for the natriuretic peptide system, we tested the effect of cell membrane-permeant cGMP, 8-BrcGMP, on the secretion of ANP. 8-BrcGMP (10⁻⁴ M) decreased atrial stroke volume (P < 0.001, n = 7, Fig. 5C) and suppressed the secretion of ANP (P < 0.001, Fig. 5A) as well as the concentration of ANP in perfusate (P < 0.05, Fig. 5D). Translocation of the ECF decreased at lower and increased at higher atrial rate (P < 0.05, Fig. 5B). 8-BrcGMP shifted the relationship between the secretion of ANP and atrial stroke volume or translocation of the ECF rightward (both P < 0.001; Fig. 5, E and G). 8-BrcGMP shifted the relationship between translocation of the ECF and atrial stroke volume leftward (P < 0.05, Fig. 5F). The effects of 8-BrcGMP on contraction and secretion of ANP were similar to those of CNP. The effect of 8-BrcGMP on atrial stroke volume was significant after 16 min, which appeared rather slowly compared with that of CNP.

Acetation by zaprinast of effect of CNP on ANP secretion. To test involvement of cGMP production in the action of CNP, a cGMP-specific phosphodiesterase inhibitor, zaprinast, was used in beating atria (Fig. 6). CNP (10⁻⁷ M, n = 9) tended to shift the relationship between secretion of ANP and translocation of the ECF rightward, although not significantly. Zaprinast (3 × 10⁻⁶ M) augmented the effect of CNP, producing a right shift of the secretion-translocation relationship (P < 0.05, n = 6). However, the relationship between secretion of ANP and translocation of the ECF was not changed significantly by zaprinast alone (n = 9).

Blockade by HS-142-1 of effects of CNP. HS-142-1 selectively and competitively blocks the effect of CNP on the biological receptors of the NPRs (27). Pretreatment with HS-142-1 (100 µg/ml, n = 3) blocked both effects of CNP (10⁻⁶ M) on the atrial stroke volume and ANP secretion (Fig. 7, A, C, and D). In the atria pretreated with HS-142-1, the positive relationship between secretion of ANP and translocation of the ECF was not affected by CNP (Fig. 7, right). HS-142-1 (100 µg/ml, n = 2) alone does not produce detectable changes in atrial contraction and ANP secretion (data not shown).

Elimination of the suppression of ANP secretion by CNP and 8-BrcGMP in the atria perfused with Ca²⁺-free buffer. To test involvement of extracellular Ca²⁺ in the suppression of ANP secretion by CNP, experiments were performed in nonbeating rat atria perfused with Ca²⁺-free buffer. In the nonbeating atria, changes in atrial DRV were proportional to the stepwise increase in intracavitary atrial pressure (Fig. 8A). An increase in atrial DRV resulted in increases in ECF translocation (Fig. 8B). Secretion of ANP was a function of DRV or translocation of the ECF (Fig. 8, C and D). CNP (10⁻⁶ M) shifted relationships between secretion of ANP and DRV or translocation of the ECF rightward (both P < 0.05; Fig. 8, C and D). In the nonbeating atria perfused with Ca²⁺-free buffer, CNP did not suppress secretion of ANP in terms of atrial DRV or translocation of the ECF (both P > 0.05; Fig. 8, E and F). Ca²⁺ omission from the...
perfusion buffer accentuated the secretion of ANP (note ANP secretion, Fig. 8, E and F, vs. Fig. 8, C and D). The suppression by CNP of ANP secretion was similarly observed in nonbeating rabbit atria (data not shown). In the same way, we tested the effects of 8-BrcGMP. 8-BrcGMP (10^{-4} M) suppressed secretion of ANP in terms of DRV and translocation of the ECF (both \( P < 0.01; \) Fig. 9, A and B). Therefore, 8-BrcGMP shifted the relationship between the secretion of ANP and translocation of the ECF rightward. In the nonbeating atria perfused with Ca^{2+}-free buffer, suppression by 8-BrcGMP of the secretion of ANP was not observed (both \( P > 0.05; \) Fig. 9, C and D). CNP (Fig. 8, A and B), and also 8-BrcGMP (data not shown), were without effect on the changes in DRV and translocation of the ECF.

Effect of CNP on cGMP production in perfused atria. CNP (10^{-6} M) elicited an increase in cGMP production in beating rabbit atria [0.64 ± 0.05 (n = 7) vs. 0.39 ± 0.05 pmol/mg protein (n = 8); \( P < 0.01; \) Fig. 10]. Pretreatment with zaprinast (3 × 10^{-6} M) tended to increase CNP-induced cGMP production of the atria compared with CNP alone, but the difference was not significant [0.77 ± 0.09 (n = 5) vs. 0.64 ± 0.05 pmol/mg protein (n = 7); \( P > 0.05 \)). No significant change in cGMP production was observed by zaprinast alone [0.27 ± 0.04 (n = 10) vs. 0.39 ± 0.05 pmol/mg protein; \( P > 0.05 \)]. In nonbeating rat atria (Fig. 8), CNP also
increased cGMP production significantly [0.64 ± 0.06 (n = 6) vs. 0.32 ± 0.03 pmol/mg protein (n = 5); P < 0.01].

Activation by CNP of particulate GC of atrial membranes. To further define the effect of CNP on cGMP production in the atria, we measured GC activity. Natriuretic peptides (all 10^-6 M) elicited an increase in cGMP production in atrial membranes (Fig. 11A). ANP increased cGMP production from the basal level (7.39 ± 0.69 to 8.54 ± 0.69 pmol·mg protein⁻¹·min⁻¹; P < 0.01, n = 12). cGMP production was increased by 1.15 ± 0.18, 3.35 ± 0.37, and 11.52 ± 1.28 pmol·mg protein⁻¹·min⁻¹ over the basal level in response to ANP, BNP, and CNP, respectively (n = 12). ANP, BNP, and CNP activated GC activity of atrial membranes, with CNP being most potent (ANP vs. BNP, P > 0.05; ANP vs. CNP and BNP vs. CNP, both P < 0.001). CNP increased cGMP production in a dose-dependent manner with an EC50 of 49 nM (n = 6, Fig. 11B). A significant increase in cGMP production over the basal level was observed at a concentration as low as 10 pM (19.24 ± 1.24 vs. 17.43 ± 1.41 pmol·mg protein⁻¹·min⁻¹; P < 0.01, Student’s t-test). The effect of CNP (10^-6 M) on cGMP production was blocked by pretreatment with HS-142-1 in a dose-dependent manner (n = 6, Fig. 11C). Suppression by HS-142-1 of the increase in cGMP production caused by CNP was significant at concentrations of 10 (P < 0.05), 100, and 500 µg/ml (both P < 0.01).
Gene expression for CNP and NPRs in rabbit atria. Figure 12 shows RT-PCR products from rabbit atrial RNA. Bands of DNA are present in the lanes corresponding to the expected size of the products for the NPRs, CNP, and ANP. Products of pituitary gland, kidney, and atrial RNA for NPRs, ANP, and CNP are also shown for positive comparison.

CNP and 8-BrcGMP increased \([\text{Ca}^{2+}]_i\) transients in single atrial myocytes. In single atrial myocytes, CNP increased \([\text{Ca}^{2+}]_i\) transients (Fig. 13). CNP (10\(^{-8}\) M, 10\(^{-7}\), and 10\(^{-6}\) M) increased the fluorescence intensity ratio (\(F_{340}/F_{380}\)) in a dose-dependent manner (Fig. 13A). \(F_{340}/F_{380}\) increased from 0.25 ± 0.03 to 0.29 ± 0.04 (\(P < 0.05\), \(n = 14\)), from 0.24 ± 0.04 to 0.32 ± 0.05 (\(P < 0.01\), \(n = 12\)), and from 0.23 ± 0.04 to 0.52 ± 0.22 (\(P > 0.05\), \(n = 9\)) in response to 10\(^{-8}\), 10\(^{-7}\), and 10\(^{-6}\) M CNP, respectively. The ratio increased significantly over the basal level in a dose-dependent manner (2.13 ± 0.49 at 10\(^{-6}\) M vs. 1.36 ± 0.09 at 10\(^{-7}\) M, \(P < 0.05\); 2.13 ± 0.49 at 10\(^{-6}\) M vs. 1.19 ± 0.07 at 10\(^{-8}\) M, \(P < 0.01\), Fig. 13B). In some myocytes, CNP resulted in a myocytic contracture with increased \([\text{Ca}^{2+}]_i\) transients. In nominally \(\text{Ca}^{2+}\)-free buffer, 10\(^{-6}\) M CNP induced no significant change in \(F_{340}/F_{380}\) (0.33 ± 0.02 vs. 0.33 ± 0.02, \(n = 10\), \(P > 0.05\), in \(\text{Ca}^{2+}\)-free buffer; 0.75 ± 0.13 vs. 0.36 ± 0.03, \(n = 10\), \(P < 0.05\), in regular \(\text{Ca}^{2+}\) buffer). 8-BrcGMP (3 × 10\(^{-4}\) M) increased \(F_{340}/F_{380}\) from 0.36 ± 0.03 to 0.40 ± 0.03 (\(P < 0.05\), \(n = 8\)). The ratio increment over baseline level was 1.13 ± 0.05 (Fig. 13C).
DISCUSSION

The present study clearly shows for the first time that CNP negatively regulates the release of ANP and atrial stroke volume. The actions of CNP on atrial dynamics and the secretion of ANP were rapid, and the effects continued during the presence of the peptide. The effects of CNP were mimicked by 8-BrcGMP, a cell-permeant analog of cGMP. The effects of CNP were accentuated by pretreatment with zaprinast and were blocked by HS-142-1, a polysaccharide inhibitor of GC-coupled NPR-A and NPR-B. CNP increased cGMP production in the perfused atria. CNP stimulated particulate GC activity of atrial membranes with the greatest potency among natriuretic peptides in the cardiac atrium. The effect of CNP on the GC activation was attenuated by HS-142-1. Together with gene expression for CNP and NPR-B, it is suggested that CNP is involved in the regulation of secretory as well as mechanical function in the endocrine atria. Therefore, it is possible to postulate that CNP suppresses the secretion of ANP and atrial dynamics via the particulate GC-cGMP pathway. In our experiments, it was shown for the first time that CNP increased [Ca\(^{2+}\)], transients of single atrial myocytes in a dose-dependent manner. 8-BrcGMP also increased [Ca\(^{2+}\)], transients. We previously showed (19) suppression of the atrial myocytic release of ANP by Ca\(^{2+}\). Therefore, the mechanism by which CNP suppresses the secretion of ANP may be related to an increase in [Ca\(^{2+}\)] transients. In the present experiments, the suppression of ANP secretion by CNP or 8-BrcGMP was eliminated by omission of Ca\(^{2+}\) from the perfusion buffer. An increase in [Ca\(^{2+}\)] transients by CNP or 8-BrcGMP of single myocytes was also eliminated by omission of Ca\(^{2+}\). However, direct suppression of myocytic ANP release by cGMP cannot be excluded in the present study.

CNP has been reported to possess minor systemic effects (43), and it has been suggested that CNP produced in tissues may function as a local modulator. The present data showing the suppression of ANP secretion by CNP and the presence of the gene tran-
scripts for CNP and its specific receptor NPR-B suggest that there is cross-talk between the natriuretic peptides in the atria. Recently, it was shown that ANP and BNP stimulate the production and secretion of CNP from bovine aortic endothelial cells (32). If this is the case in the atrial endocardium, there is a feedback control between members of the natriuretic peptide family: a positive accentuation of CNP production and secretion by ANP and BNP and a negative suppression of ANP secretion by CNP. The minimal effective concentration of CNP was 300 nM for the suppression of ANP secretion and atrial dynamics in the beating atria and as low as 10 pM for the cGMP production in atrial membranes. The EC_{50} of CNP for cGMP production was estimated at 49 nM in atrial membranes, which is comparable with the previous report in cultured cells (24). Therefore, CNP may play a physiological role in the endocrine atria. Previously, it was shown that ANP inhibits its own secretion via NPR-A (26, 31). From the finding that HS-142-1 increases the secretion of ANP in cultured atrial myocytes (31) and in vivo (26), those authors suggested a negative regulation by endogenous ANP of its own secretion. Leskine et al. (26), however, could not find an inhibition by CNP of ANP secretion in vivo. The difference between that study and the present data obtained from a rabbit atrial preparation in vitro may be related to the responsiveness of NPR-B to CNP in their in vivo rat model. In the present experiments, HS-142-1 was without effect on ANP secretion, which suggests that interstitially released ANP may not be as potent as CNP in the suppression of ANP secretion in the atrium. This is consistent with the findings that ANP and BNP are not as potent as CNP in the activation of particulate GC of the atrial membranes (Fig. 11A) and that the expression of mRNA for NPR-B is much higher than that for NPR-A in the atria (2). Alternatively, the concentration of HS-142-1 may not be enough to antagonize the effect of the released ANP in the present study.

An increase by 8-Br-cGMP of [Ca^{2+}]_i transients is consistent with previous data (13, 22, 34). Kirstein et al. (22) reported an increase in Ca^{2+} current by cGMP in human atrial myocytes. Similarly, Han et al. (13) and Ono and Trautwein (34) showed that cGMP increases L-type Ca^{2+} current in ventricular myocytes. However, our data contrast with those of another report showing a decrease in Ca^{2+} current by cGMP in cardiac myocytes (28). The mechanism by which CNP increases [Ca^{2+}]_i transients in the present experiment may be closely related to the production of cGMP. Therefore, it is possible to postulate that CNP negatively regulates atrial myocyte release of ANP by increasing [Ca^{2+}] via NPR-B-cGMP signaling. It is of interest that 8-Br-cGMP is not as effective on intracellular Ca^{2+} as CNP. The reason for the difference may be related to the slower intracellular accumulation of 8-Br-cGMP through the cell membrane compared with the cGMP production via GC activation by CNP. The present result showing suppression by 8-Br-cGMP of ANP secretion is consistent with previous reports (15, 31) but contrasts with other studies reporting a lack of effect of 8-Br-cGMP on ANP secretion (16, 36).

The suppression by CNP of atrial stroke volume and pulse pressure contrasts with those of previous reports in dog atria (2, 14). Beaulieu et al. (2) and Hirose et al. (14) reported that CNP induces a positive inotropic effect in dog atria. They suggested that this effect of CNP is mediated via NPR-B signaling. Beaulieu et al. (2) further suggested an activation of the Ca^{2+} channel by CNP. Hirose et al. (14) suggested that the positive inotropic effect of CNP could be caused by inhibition of cGMP-inhibited phosphodiesterase. These results, together with the present data showing a negative inotropic effect, suggest that CNP may produce diverse effects on myocardial contractility after activation of the GC signal transduction pathway. The reasons for the difference of the effect of CNP on atrial dynamics are not clear at present. Because the intracellular cGMP causes a concentration-dependent biphasic contractile response (30), the concentration of CNP used in previous studies could possibly be related to this problem. However, this is not the case in the present experiments. CNP (10^{-8} M), which was used in a similar concentration to that of the previous study (14), showed a tendency to decrease atrial dynamics. The differences in the atrial preparation and animal species used for the experiments may account for this discrepancy. The mechanism by which CNP suppresses atrial dynamics may be related to increased cGMP production and reduced myofilament response to Ca^{2+}. This is consistent with the suggestion of Shah et al. (39), who showed that cGMP reduces the myofilament response to Ca^{2+} in cardiac myocytes.

From the present results it is proposed that the activation of NPR-B by CNP produces two intracellular
signals that have distinct effects in the atrium: a suppression of the myocytic contractility by increased cGMP, and an increase in [Ca$^{2+}$], (Fig. 1). Both of the above-described effects of CNP seem to be related to changes in the two-step sequential mechanism that we have proposed for the regulatory mechanism of the ANP secretion from the atria (4, 5). In the proposed two-step sequential mechanism, first, ANP is released from myocytes into the surrounding paracellular space, and second, translocation of the ANP-containing ECF into the bloodstream is induced by atrial contraction. Therefore, the increased [Ca$^{2+}$], possibly caused by cGMP, and also cGMP acting directly itself, may be related to suppression of myocytic release of ANP, the first step of ANP secretion. Because the second step of ANP secretion, i.e., translocation of the ECF with released ANP, has been shown to be regulated by atrial dynamics, atrial stroke volume, and atrial rate (5), it might be expected that translocation of the ECF would be suppressed by CNP. Even though atrial dynamics were suppressed by CNP in the present study, translocation of the ECF was not decreased. CNP decreased the concentration of ANP in terms of ECF translocation and also shifted the relationship between the secretion of ANP and translocation of the ECF rightward. These findings indicate that CNP suppresses the myocytic release of ANP into the surrounding paracellular space (4).

The present data showing CNP and NPR-B gene expression in the cardiac atria are consistent with the data previously reported. mRNA for CNP has been reported to be expressed in rat (46) and human (11) atria. Gene expression for NPR-B has also been reported in rat (33) and dog (2) atria. Furthermore, Doyle et al. (8) showed the presence of NPR-B protein in rat atria.

In summary, the data indicate that CNP inhibits mechanically stimulated release of ANP and atrial dynamics via NPR-B-cGMP signaling and that an increase in [Ca$^{2+}$], is involved in the pathway of NPR-B-cGMP signaling in suppression of myocytic release of ANP.

Perspectives

A proposed two-step sequential mechanism for the regulation of mechanically stimulated ANP secretion allows us to dissect functional steps of the ANP secretion pathway. We interpret the concentration of ANP in perfusate in terms of ECF translocation and the relationship between ANP secretion and ECF translocation as mirroring the concentration of released ANP in the paracellular space. Therefore, the technique is a unique method capable of analyzing the rate of myocytic release of ANP in atrial tissue. Although an activation by CNP of NPR-B-cGMP signaling inhibits mechanically stimulated release of ANP, possibly via increase in [Ca$^{2+}$], the mechanism for the signal transduction between the activation of the CNP system and increase in [Ca$^{2+}$], and, subsequently, inhibition of myocytic release of ANP is yet to be defined. The present data, together with previous reports (2, 14), suggest that diverse signal transduction mechanisms are involved in the regulation of atrial myocardial contractility by an activation of NPR-B-cGMP signaling.

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