C-Type Natriuretic Peptide Neuromodulates Independently of Guanyllyl Cyclase Activation

George J. Trachte, James G. Drewett

Abstract Of the four endogenous members of the natriuretic peptide family, only atrial natriuretic peptide has been demonstrated to have neuromodulatory effects. This study compares the neuromodulatory effects of atrial natriuretic peptide and a recently identified natriuretic peptide, C-type natriuretic peptide, in the rabbit isolated vas deferens. The ability of these peptides to alter cyclic nucleotide concentrations was assessed to determine the potential contribution of either cyclic AMP or cyclic GMP to the observed responses. The central hypothesis tested was that C-type natriuretic peptide modulates neurotransmission via an interaction with a guanylyl cyclase. C-type natriuretic peptide inhibited both purinergic and adrenergic neurotransmission in a concentration-dependent manner but failed to alter either cyclic GMP or cyclic AMP concentrations. Maximal inhibitory effects of C-type natriuretic peptide averaged 35±4% for purinergic and 49±7% for adrenergic neurotransmission. Atrial natriuretic peptide not only attenuated both purinergic and adrenergic neurotransmission but also increased cyclic GMP concentrations. C-type natriuretic peptide probably inhibited the release of the neurotransmitters because it failed to alter contractions to exogenously administered norepinephrine or ATP, the two putative neurotransmitters. These results suggest that the C-type natriuretic peptide receptor, guanyllyl cyclase B, is not present in rabbit vas deferens and that C-type natriuretic peptide suppresses peripheral sympathetic neurotransmission independently of guanlyl cyclase activation. (Hypertension. 1994;23:38-43.)

Key Words • natriuretic peptides • neuroregulators • receptors, purinergic • receptors, adrenergic • nucleotides, cyclic • guanosine cyclic monophosphate • adenosine cyclic monophosphate

Four endogenous members of the natriuretic peptide family have been identified since the classic demonstration of deBold et al1 of natriuresis in response to the injection of atrial extracts in rats. In addition to atrial natriuretic peptide (ANP), brain natriuretic peptide, C-type natriuretic peptide (CNP), and urodilatin have been discovered.2-4 The natriuretic peptides all produce natriuresis and hypotension.5 Among other actions, ANP suppresses adrenergic neurotransmission, an action potentially related to hypotensive activities.6 We and others have defined extensively the neuromodulatory activity of ANP.7-10 It reduces adrenergic neurotransmitter release from adrenergic nerves7 and can reduce the contractile effects of adrenergic neurotransmitters,11,12 such as norepinephrine, depending on the tissue being examined. The neuromodulatory activity of ANP was independent of changes in cyclic GMP (cGMP) concentrations and could be mimicked by a compound specific for the guanylyl cyclase–uncoupled “clearance” receptor.9,10 Pertussis toxin also eliminated the neuromodulatory effect of ANP without affecting cGMP production.9,10 Thus, prior results with ANP predict that the more recently discovered natriuretic peptides should act as inhibitory neuromodulators by a mechanism independent of cGMP.

The most recently discovered natriuretic peptide, CNP, is reported to be produced primarily in neuronal tissue3,13,14; therefore, it is likely to have effects on neurotransmission. Despite this rationale for a neuromodulatory action, CNP has not been reported thus far to alter neurotransmission. This study attempts to define neuronal effects of CNP in a peripheral tissue containing adrenergic neurons, the vas deferens, and to compare its actions with those of ANP. This report focuses on the relation between CNP effects on cyclic nucleotide concentrations and neurotransmission. The results indicate that CNP produces neuromodulatory effects in the absence of changes in cGMP concentrations. These data suggest that the biologic activity of CNP is not limited to tissues possessing the CNP receptor guanlyl cyclase-B (GC-B).15

Methods

A total of 20 rabbits were obtained from local breeders and used for the studies reported. Institutional guidelines were followed to limit distress to the animals as much as possible. Mixed-breed rabbits were anesthetized with sodium pentobarbital (50 mg/kg) injected into an ear vein. The vas deferentia were removed and placed in Krebs-bicarbonate buffer. Each vas deferens was desheathed and cut into 2-cm lengths. These tissues were placed in organ baths containing warmed (37°C) Krebs-bicarbonate buffer gassed with 95% oxygen and 5% carbon dioxide. The vas deferens was passed through two platinum ring electrodes and connected to a Grass FT03c force transducer and a glass support with silk suture. One gram of resting force was maintained on each vas deferens. The electrodes were positioned 1.5 cm apart. Electrical stimulation was used to activate adrenergic nerves in the preparation and was accomplished with a Grass S44 stimulator set to deliver a 70-V pulse (10 V measured at the electrodes) at a frequency of 2 Hz with a 1-millisecond pulse duration and 10-millisecond

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delay between pulses. These parameters result in contractions sensitive to either tetrodotoxin (100 ng/mL) or appropriate purinergic and adrenergic antagonists. The electrically induced contraction is composed of both a twitch contraction of a purinergic nature and a tonic contraction of an adrenergic nature. The vasa were stimulated for 10 seconds every 2 minutes. A 2-hour equilibration time in the organ baths was allowed to reestablish ionic equilibrium. After equilibration, each vas deferens was stimulated until stable contractions were obtained, normally requiring 30 minutes of stimulation. Once contractions were reproducible, either ANP or CNP was added in log increments at 6-minute intervals.

Cyclic Nucleotide Measurements

Tissues used for cyclic nucleotide measurements were treated identically except that they were neither electrically stimulated nor stretched. They were bathed in buffer containing isobutylmethylxanthine (2.5 mmol/L) to suppress phosphodiesterase activity. The tissues were removed from the bath after a 6-minute exposure to ANP, CNP, or vehicle and homogenized in ethanol using an Ultraturax T-25 homogenizer (Janke-Kunkel, Staufen, Germany). The homogenate was spun at 5000 gravitational equivalents in a Jouan CR 4.11 centrifuge, and supernatants were dried in a Sybron/Brinkman SC240 sample concentrator. The cyclic nucleotides were reconstituted in radioimmunoassay buffer and analyzed for cGMP and cyclic AMP (cAMP) concentrations using Amersham kits (TRK.500 and TRK.432, Arlington Heights, Ill.). Concentrations were normalized to wet weight of each preparation.

Postfunctional Effects of C-Type Natriuretic Peptide

The electrically induced contraction of the vas deferens involves both the release of neurotransmitter from neurons and the contractile effect of neurotransmitters on smooth muscle of the preparation. Therefore, the effect of an agent could involve either an alteration of neurotransmitter release or an alteration of smooth muscle responsiveness to neurotransmitters. The latter possibility was tested by incubating vasa deferentia with 100 nmol/L CNP added 2 minutes before generation of a concentration-response curve to ATP or norepinephrine, the two putative sympathetic neurotransmitters in the vas deferens. A contralateral vas deferens was treated with the CNP vehicle (ie, Krebs-bicarbonate buffer) before the ATP or norepinephrine additions. The responses of the two vasa deferentia were compared to test for a postfunctional effect of CNP.

Effects of Pertussis Toxin

A prior study established that ANP failed to alter neurotransmission in the presence of pertussis toxin (100 ng/mL); therefore, we tested the effect of pertussis toxin on CNP neuromodulatory effects to assess if it acted similarly to ANP. Tissues were incubated with pertussis toxin or its vehicle (Krebs-bicarbonate buffer) for various periods of time and then were stimulated and exposed to CNP.

Materials

Chicken CNP was obtained from Peninsula Laboratories, Belmont, Calif, and human ANP was purchased from Sigma Chemical Co, St Louis, Mo. Chicken CNP was used because it was the only CNP commercially available at the start of these studies. We have confirmed in subsequent experiments that human CNP behaves similarly to chicken CNP. Pertussis toxin, norepinephrine, and ATP were purchased from Sigma.
FIG 2. Line graph shows effect of atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP) on adrenergic (tonic) contractions of vas deferens. Values are mean±SEM; N indicates the number of preparations per group. Both compounds significantly suppressed contractions at concentrations exceeding 0.1 nmol/L for ANP and 1 nmol/L for CNP. Curves were significantly different, as indicated by asterisks, when compared by analysis of variance (P<.01), with CNP resulting in a slightly less potent effect than ANP. CON indicates control.

Adrenergic contractions. Adrenergic contractions were suppressed significantly at ANP concentrations of 1, 10, and 100 nmol/L (P<.01), and CNP significantly attenuated contractions at concentrations of 10 and 100 nmol/L (P<.01). The response to 100 nmol/L ANP was maximal, with higher ANP concentrations failing to augment the response further (data not shown). The response to ANP was significantly greater than that to CNP (P=.009), but the slopes did not differ significantly. The EC50 for ANP (2.0±0.6 nmol/L) differed from that for CNP (14.5±4.7 nmol/L) (P<.01). These results indicate that CNP is an adrenergic neuromodulator, although it is less effective than ANP.

The ANP-induced concentration-dependent augmentation of cGMP concentrations is shown in Fig 3. In contrast to ANP, CNP failed to influence cGMP levels at any concentration tested. Initial cGMP concentrations averaged 25±10 and 29±13 fmol/mg vas deferens in preparations treated with ANP or CNP, respectively. Although CNP had no significant effect on cGMP concentrations in the vas deferens, ANP concentrations of 10 and 100 nmol/L did (P<.05). The two curves differed significantly (P=.007), as did their slopes (P=.01). The ANP effect was concentration dependent. These data suggest the absence of GC-B, the guanylyl cyclase responsive to CNP, and the presence of the ANP-selective guanylyl cyclase receptor GC-A. The incremental increases in cGMP concentrations in response to ANP were sustained in the presence of pertussis toxin (100 ng/mL for 7 hours; data not shown).

The effect of CNP on cAMP concentrations with and without pertussis toxin is shown in Fig 4. The lowest concentration of CNP tended to suppress cAMP concentrations, but no significant effect was observed at any CNP concentration. We observed essentially the same effect of ANP in this tissue in a previous study. Pretreatment with pertussis toxin (100 ng/mL) for 7 hours did not significantly alter these responses. These results indicate no correlation between CNP effects on cAMP concentrations and neurotransmission.

FIG 3. Line graph shows effect of atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP) on cyclic GMP (cGMP) concentrations of vas deferens. All values are mean±SEM; number of preparations per group is shown in parentheses. ANP significantly augmented cGMP concentrations, whereas CNP did not. Asterisks indicate that curves differed significantly when compared by analysis of variance (P<.01). CNP failed to have an influence on cGMP concentrations at any concentration tested. CON indicates control.

FIG 4. Line graph shows C-type natriuretic peptide (CNP) effects on cyclic AMP (cAMP) concentrations in the presence of pertussis toxin (PTX, 100 ng/mL) or its vehicle (VEH, Krebs' buffer). All values are mean±SEM; N indicates the number of preparations in each group. CNP failed to alter cAMP concentrations. The tendency to suppress cAMP concentrations at 1 nmol/L CNP was eliminated by pertussis toxin, but the effect only approached statistical significance (P=.07). The curves were not statistically different. CON indicates control.
The effect of pertussis toxin on purinergic and adrenergic neuromodulatory effects of CNP is shown in Figs 6 and 7. Pertussis toxin treatment for 7 hours tended to blunt inhibitory effects of CNP on purinergic neurotransmission, but no statistically significant effect was present (P=0.31). The action of CNP was concentration dependent in the presence or absence of pertussis toxin. The CNP effect on adrenergic neurotransmission was significantly blunted by the 7-hour pretreatment with pertussis toxin (100 ng/mL, P<0.05). The effect of CNP was still concentration dependent in the presence or absence of pertussis toxin, but the magnitude of the neuromodulatory effect was attenuated by the pertussis toxin.

The results suggest that CNP acts at a receptor other than GC-B to suppress neurotransmission independently of cGMP concentrations. One possible interpretation of the inability of CNP to activate guanylyl cyclase involves an absence of GC-B in rabbit vas deferens. However, the results also could derive from an inability of chicken CNP to act on GC-B in a different species, the rabbit. This possibility was tested by examining CNP effects on cGMP concentrations in the rabbit kidney, a tissue containing both GC-A and GC-B.19 As shown in Fig 8, CNP elevated cGMP concentrations of kidney slices approximately fivefold at concentrations of 1 nmol/L. This represented a statistically significant elevation, indicating that chicken CNP has the capacity to stimulate guanylyl cyclase activity in the rabbit.

Discussion

The novel findings of this study are the following: (1) CNP suppresses both purinergic and adrenergic neurotransmission, confirming its suspected role as a neuromodulator; (2) CNP acts as a neuromodulator in the
are mean±SEM; N indicates the number of preparations per group. CNP significantly increased cGMP contents (expressed as femtomoles per milligram wet weight) at a concentration of 1 nmol/L. Asterisk indicates a difference from control (CON) (P<.05).

absence of detectable changes in cGMP concentrations; (3) peripheral sympathetic nerves, at least in the vas deferens, appear to be devoid of GC-B because CNP did not increase cGMP concentrations in this tissue; (4) CNP does not alter contractile responses to either ATP or norepinephrine in this reproductive smooth muscle, suggesting that it acts prejunctionally to suppress neurotransmission; and (5) CNP attenuates neurotransmission via a pertussis toxin-sensitive mechanism. The finding that CNP is a neuromodulator is novel but not surprising because it has been touted as a neuromodulator since its discovery in the brain. These data confirm the potent for CNP to serve a physiological neuromodulatory function, although it is slightly less effective than ANP. The absence of a guanylyl cyclase response to CNP is surprising in that CNP activity is believed to be limited to tissues containing GC-B receptors. This deficiency of a guanylyl cyclase response to CNP suggests that this tissue lacks GC-B receptors. The presumably prejunctional effects of CNP to suppress neurotransmitter release were suspected because this is the mechanism ANP and analogues use to suppress neurotransmission in the vas deferens.10,11 The major finding of this investigation is that CNP suppresses sympathetic neurotransmission similarly to ANP but in the complete absence of a change in cGMP concentrations. These data are consistent with a neuromodulatory pathway distinct from guanylyl cyclase–linked receptors.

Prior work in the rabbit vas deferens and other tissue innervated by the sympathetic nervous system9,10 showed that ANP suppressed neurotransmission by a prejunctional mechanism sensitive to pertussis toxin and mediated by the GC-uncoupled "clearance" receptor.20 An ANP derivative specific for the clearance receptor, cANF, also mimicked the effect of ANP in suppressing neurotransmission.10 CNP could act through a similar mechanism inasmuch as it binds to the clearance receptor in addition to GC-B.11 The simplest scenario encompassing all these results involves a common neuromodulatory receptor for cANF, CNP, and ANP. The clearance receptor is the only natriuretic peptide receptor identified yet to bind with all three of these peptides; therefore, this receptor, or another unidentified natriuretic peptide receptor with a similar pharmacologic profile, is most likely the neuromodulatory receptor for natriuretic peptides.

This work represents the first report of an endogenous natriuretic peptide altering neurotransmission in the complete absence of a change in cGMP concentrations. The results are consistent with numerous recent reports suggesting a biologic function for natriuretic peptides not coupled to guanylyl cyclase in platelets,21 thyroid,22 heart,23 and adrenal.24 This accumulating evidence argues against the prevailing perception that ANP acts solely via generation of cGMP.

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References

1. deBold AJ, Borenstein HB, Veres T, Sonneborn H. A rapid and potent natriuretic response to intravenous injection of atrial myocardi- 
2. Sudoh T, Kangawa K, Minamino N, Matsuo H. A new natriuretic 
3. Sudoh T, Minamino N, Kangawa K, Matsuo H. C-type natriuretic 
4. Schulz-Knappe P, Fursmann K, Herbst F, Hock D, Pipkorn R, 
5. Lang CC, Choy A-MI, Struthers AD. Atrial and brain natriuretic 
6. Debinski W, Kuchel O, Busi NT. Atrial natriuretic factor is a new 
7. Nakamura M, Inagami T. Atrial natriuretic factor inhibits norepi-
8. Holtz J, Sommer O, Bassenge E. Inhibition of sympathoadrenal 
9. Tomura Y, Yamagata T, Hisa H, Satoh S. Effects of atrial natriu-
10. Johnson DG, Trachte GJ, Drewett JG. Neurmodulatory effect of 
11. Johnson DG, Trachte GJ, Drewett JG. Neurmodulatory effect of 
12. Tomura Y, Yamagata T, Hisa H, Satoh S. Effects of atrial natriu-
13. Tomura Y, Yamagata T, Hisa H, Satoh S. Effects of atrial natriu-
15. Suga S-l, Nakao K, Ih0 H, Komatsu Y, Ogawa Y, Hama N, Imura 
17. Suga S-l, Nakao K, Ih0 H, Komatsu Y, Ogawa Y, Hama N, Imura 
18. Johnson DG, Trachte GJ, Drewett JG. Neurmodulatory effect of 
19. Johnson DG, Trachte GJ, Drewett JG. Neurmodulatory effect of 
20. Johnson DG, Trachte GJ, Drewett JG. Neurmodulatory effect of 
21. Johnson DG, Trachte GJ, Drewett JG. Neurmodulatory effect of 
22. Johnson DG, Trachte GJ, Drewett JG. Neurmodulatory effect of 
23. Johnson DG, Trachte GJ, Drewett JG. Neurmodulatory effect of 
24. Johnson DG, Trachte GJ, Drewett JG. Neurmodulatory effect of 
25. Johnson DG, Trachte GJ, Drewett JG. Neurmodulatory effect of 
26. Johnson DG, Trachte GJ, Drewett JG. Neurmodulatory effect of 
27. Johnson DG, Trachte GJ, Drewett JG. Neurmodulatory effect of 
28. Johnson DG, Trachte GJ, Drewett JG. Neurmodulatory effect of 
29. Johnson DG, Trachte GJ, Drewett JG. Neurmodulatory effect of 
30. Johnson DG, Trachte GJ, Drewett JG. Neurmodulatory effect of 

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