C-Type Natriuretic Peptide Neuromodulates Independently of Guanylyl Cyclase Activation

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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, guanylyl cyclase B, is not present in rabbit vas deferens and that C-type natriuretic peptide suppresses peripheral sympathetic neurotransmission independently of guanylyl cyclase activation. (Hypertension. 1994;23:3843.)

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,1112 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 tissue2,3,14; 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 guanylyl 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 vasa 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...
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 constituted in radioimmunoassay buffer and analyzed for cGMP and cyclic AMP (cAMP) concentrations using Amer sham kits (TRK.500 and TRK.432, Arlington Heights, Ill). Concentrations were normalized to wet weight of each preparation.

Postjunctional 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 postjunctional 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.

![Graph showing effect of atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP) on purinergic (twitch) contractions of vas deferens. All points are means with bars representing standard errors. N indicates the number of preparations in each group. There were no significant differences between groups, although significant depressions of contractile force were observed in both groups at concentrations exceeding 1 nmol/L. CON indicates control. ](image)

Statistical Analysis

Curves were compared by analysis of variance for repeated measures. Individual values were compared by Student's t test with Dunnett's correction for multiple comparisons. A value of P<.05 was required for statistical significance. The concentration of a natriuretic peptide producing a half-maximal response (EC50) was used as an indicator of potency. Maximal responses to ANP were used to define maximal responses to natriuretic peptides.

Results

As shown in Fig 1, both ANP and CNP suppressed twitch (purinergic) contractions of the vas deferens in response to electrical stimulation. The basal force generated by this contraction averaged 1.30±0.22 and 1.26±0.23 g in the tissues exposed to ANP and CNP, respectively. These values did not differ significantly. Both ANP and CNP significantly suppressed electrically induced contractions at concentrations of 10 and 100 nmol/L (P<.001 for ANP and P<.05 for CNP). Higher concentrations of the natriuretic peptides failed to produce greater effects on purinergic neurotransmission (data not shown). The curves were not significantly different when compared by analysis of variance. Furthermore, the slopes of the curves did not differ significantly, yielding half-maximal responses at concentrations (EC50 values) of 2.5±0.9 and 6.9±2.9 nmol/mL, respectively. The effects of both ANP and CNP were concentration dependent. These results indicate that CNP inhibits purinergic neurotransmission similarly to ANP.

Both ANP and CNP also suppressed adrenergic neurotransmission, as shown in Fig 2. Initial contractions averaged 0.46±0.08 and 0.65±0.17 g for preparations treated with ANP and CNP, respectively. These values did not differ significantly. Both peptides produced concentration-dependent suppressions of adren-
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.

Effect of ANP in this tissue in a previous study.18 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 5 presents the contractile effects of ATP and norepinephrine in the presence and absence of CNP (100 nmol/L). ATP caused a concentration-dependent
concentration that was unaffected by CNP. Norepinephrine contracted the vas deferens to a greater degree than ATP in a concentration-dependent manner unaffected by CNP. These results indicate that CNP fails to influence smooth muscle contractions to either putative neurotransmitter, suggesting that CNP acts prejunctionally to reduce neurotransmitter release from adrenergic neurons.

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 \(P=.31\). 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 \(100 \text{ ng/mL}, P<.05\). 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. As shown in Fig 8, CNP elevated cGMP concentrations in 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...
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 potential 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. 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 system showed that ANP suppressed neurotransmission by a prejunctional mechanism sensitive to pertussis toxin and mediated by the GC-uncoupled "clearance" receptor. An ANP derivative specific for the clearance receptor, cANF, also mimicked the effect of ANP in suppressing neurotransmission. CNP could act through a similar mechanism inasmuch as it binds to the clearance receptor in addition to GC-B. 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, thyroid, heart, and adrenal. This accumulating evidence argues against the prevailing perception that ANP acts solely via generation of cGMP.

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