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Natriuretic peptides like NO facilitate cardiac vagal neurotransmission and bradycardia via a cGMP pathway

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Herring, Neil, Junaid A. B. Zaman, and David J. Paterson. Natriuretic peptides like NO facilitate cardiac vagal neurotransmission and bradycardia via a cGMP pathway. *Am J Physiol Heart Circ Physiol* 281: H2318–H2327, 2001.—We tested the hypothesis that natriuretic peptide receptors (NPRs) that are coupled to cGMP production act in a similar way to nitric oxide (NO) by enhancing acetylcholine release and vagal-induced bradycardia. The effects of enzyme inhibitors and channel blockers on the action of atrial natriuretic peptide (ANP), brain-derived natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) were evaluated in isolated guinea pig atrial-right vagal nerve preparations. RT-PCR confirmed the presence NPR B and A receptor mRNA in guinea pig sinoatrial node tissue. BNP and CNP significantly ($P < 0.05$) enhanced the heart rate (HR) response to vagal nerve stimulation. CNP had no effect on the HR response to carbamylcholine and facilitated the release of [^{3}H]acetylcholine during atrial field stimulation. The particulate guanylyl cyclase-coupled receptor antagonist HS-142–1, the phosphodiesterase 3 inhibitor milrinone, the protein kinase A inhibitor H89, and the N-type calcium channel blocker ω-conotoxin all blocked the effect of CNP on vagal-induced bradycardia. Like NO, BNP and CNP facilitate vagal neurotransmission and bradycardia. This may occur via a cGMP-PDE3-dependent pathway increasing cAMP-PKA-dependent phosphorylation of presynaptic N-type calcium channels.

nitric oxide; autonomic nervous system; acetylcholine; heart rate

THE NATRIURETIC PEPTIDE FAMILY is made up of at least four distinct peptides: atrial natriuretic peptide (ANP), brain-derived natriuretic peptide (BNP), C-type natriuretic peptide (CNP), and D-type natriuretic peptide (DNP). ANP is secreted from the atria in response to myocardial stretch and acts as a cardiac hormone producing natriuresis and vasorelaxation (17). BNP produces similar effects to ANP and is synthesized and secreted in the atria and ventricle (26, 33). Plasma concentrations of BNP are used as a clinical marker for left ventricular dysfunction in conditions of volume overload (39). CNP is found within the vascular endothelium (17) but also in the rat heart (47) and the human ventricle (48). A structurally similar peptide named DNP has been identified in human plasma (40).

Although the role of natriuretic peptides in regulating effective circulating volume is well characterized, their role in the autonomic control of heart rate (HR) is controversial. Natriuretic peptides bring about their effects by stimulating the production of cGMP via particulate guanylyl cyclase-coupled receptors (NPRs) (10). ANP augments cardiac parasympathetic nerve activity (52) in humans and the negative chronotropic response to efferent vagal nerve stimulation following cardiac denervation in the anesthetized rat (2). In vitro, ANP has no effect on the HR response to muscarine, suggesting that ANP may act presynaptically to augment vagal neurotransmission (4). However, others did not observe this (1) and found no evidence that ANP modulates cardiac acetylcholine release (23). The effects of BNP and CNP on vagal control of cardiac excitability have not been investigated.

Other messengers that raise cGMP levels, such as nitric oxide (NO), do modulate the control of cardiac pacemaking. Independent of the autonomic nervous system, NO causes tachycardia due to a cGMP-dependent increase in the hyperpolarization-activated current (I_f) (24, 34). A similar tachycardia accompanied by an increase in the rate of diastolic depolarization has been reported to occur with application of CNP in vitro, although how this is brought about is not known (5, 22). Presynaptically, inhibition of neuronal NO synthase reduces the magnitude of vagal-induced bradycardia (20), whereas NO donors facilitate the release of acetylcholine to augment the HR response to vagal nerve stimulation (21). The mechanism underlying this response probably involves cGMP inhibition of phosphodiesterase 3 (PDE3) increasing cAMP-protein kinase A (PKA)-dependent phosphorylation of presynaptic N-type calcium channels. This pathway may augment vagal-induced bradycardia by promoting calcium influx and vesicular release of acetylcholine (21).

We therefore tested two hypotheses. First, is the increase in baseline HR caused by natriuretic peptides sensitive to blockers of I_f? Second, do natriuretic peptides facilitate vagal neurotransmission and bradycardia by a similar presynaptic cGMP-dependent pathway to NO?

METHODS

Experiments conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes

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of Health (NIH Publication No. 85-23, Revised 1996) and the United Kingdom's Animals (Scientific Procedures) Act 1986. Experiments were performed under British Home Office Project License PPL 30/1133.

Isolated guinea pig sinoatrial node-right vagus nerve preparation. Adult (400–500 g) female guinea pigs (*Cavia porcellus*, Dunkin Hartley, $n = 77$) were killed by cervical dislocation and exsanguinated. The thorax was opened, and ventricles were removed so that heparinized Tyrode's solution (1,000 U/ml) could be rapidly perfused into both atria. The heart was removed with the rib cage and mediasternum and placed in a dissecting dish with Tyrode's solution aerated with 95% O_2-5% CO_2 at room temperature. Any remaining ventricle and both lungs were carefully removed and the atria and mediasternum dissected free from the thorax. The right vagus was carefully separated from the carotid artery and tied. Sutures (Ethicon 5-0 silk) were placed at the lateral edges of the two atria. The preparation was then transferred to a preheated (37 ± 0.2°C), continuously oxygenated, water-jacketed organ bath containing 60 ml of Tyrode's solution. The atria were mounted vertically with the suture in the left atrium attached to a stainless steel hook, and the right atrium was attached to an isometric force transducer (HDE F30) connected to an amplifier. Data were acquired on a Power Macintosh 8500 computer using a Biopac Systems MP100 data acquisition system and Acqknowledge 3.5 software. Beating rate was triggered from contraction, and the signals were displayed in real time. Data were stored on optical disk for offline analysis.

Before each protocol was started, the mounted atria were kept in Tyrode's solution for at least 60 min until their beating rate stabilized (±5 beats/min, over 20 min). The Tyrode's solution in the organ bath was replaced approximately every 30 min throughout each protocol. The vagus nerve was stimulated at 1, 3, and 5 Hz, 10–15 V, 1-ms pulse duration for 30 s every minute until three consistent responses were obtained. We have previously shown that all HR changes from vagal nerve stimulation are completely abolished by hyoscine in this preparation and are therefore due to release of acetylcholine (42). A control experiment showed that the HR response to vagal nerve stimulation remained constant (±1 beats/min at 5 Hz) over a 2-h period. Drugs were applied directly to the organ bath and incubated until a consistent HR response to vagal nerve stimulation was obtained.

Measuring [^{3}H]acetylcholine release to field stimulation from isolated guinea pig right atrial preparations. Adult (400–500 g) female guinea pigs ($n = 10$) were killed and the right atria removed as described above. The preparation was then transferred to a preheated (37 ± 0.2°C), continuously oxygenated, water-jacketed organ bath containing 2 ml of Tyrode's solution, where the atrium was pinned flat between two parallel silver stimulating electrodes 10 mm apart. The methods for radiolabeling of cholinergic transmitter stores so that acetylcholine release could be quantified have been reported by us previously (21) and are similar to those originally devised by Wetzel and Brown (49–51) and subsequently modified by Seebeck et al. (44). After a 30-min equilibration period (where the Tyrode's solution was replaced every 15 min), the atrium was stimulated at 10 Hz (20 V, 1-ms pulse duration) for 1 min and then again after another minute to stimulate acetylcholine turnover. The preparation was incubated for 30 min with [^{3}H]choline chloride (5 μCi, Amersham), during which the atrium was stimulated at 10 Hz for 10 s every 30 s to incorporate the [^{3}H]choline chloride into the parasympathetic transmitter stores. Tyrode's solution containing 50 μM hemicholinium 3 was used after the incubation period to reduce reuptake of radioactively labeled transmitter. Excess [^{3}H]choline was washed from the preparation by superfusing for 30 min at a rate of 2 ml/min with Tyrode's solution. Superfusion was then stopped, and the bath solution was replaced every 3 min with a 0.5-ml sample being taken at every change of solution. This sample was added to 4.5 ml of scintillation fluid (Ecoscint A, National Diagnostics), and the amount of radioactivity in each sample (disintegrations per minute) was measured using a liquid scintillation counter (Tri-carb 2000CA, Packard). After 28 min and again after 43 min, the atrium was stimulated at 10 Hz for 1 min. At the end of the experiment, the atrium was immersed overnight in ≈4 U/ml papain, and the radioactivity contained in the extract was determined. ^{3}H outflow was expressed as a percentage of the total radioactivity in the atrium at the end of the experiment and released after superfusion.

Solutions and drugs. The Tyrode's solution contained (in mM) 120 NaCl, 4 KCl, 2 $MgCl_2$, 25 $NaHCO_3$, 2 $CaCl_2$, 0.1 Na_2HPO_4, and 11 glucose. The solution was aerated with 95% O_2-5% CO_2 (pH 7.4), and its temperature was continuously monitored (Digitron 1408-K gauge) and kept at 37 ± 0.2°C.

ANP factor 1-28, human (Calbiochem), BNP factor 1-32, human (Calbiochem), and CNP, human/porcine (Calbiochem) were used in concentrations up to 500 nM. All human natriuretic peptides have been shown to mediate cGMP-dependent effects in guinea pig tissue (e.g., Ref. 37). To determine whether any change in baseline HR were mediated by I_f, these experiments were repeated in the presence of the I_f blockers cesium chloride (14) (2 mM, Sigma) or 4-(*N*-ethyl-*N*-phenylamino)-1,2-dimethyl-6-(methylamino)-pyrimidinium chloride (8) (ZD-7288, 1 μM, Tocris Cookson). The effects of natriuretic peptides on the HR response to vagal nerve stimulation were compared with those of the stable analog of acetylcholine carbamylcholine (30, 60, and 90 nM, Sigma) to determine whether the effects were pre- or postsynaptic. Concentrations were chosen to produce similar degrees of bradycardia to 1, 3, and 5 Hz vagal nerve stimulation. The microbial polysaccharide HS-142–1 (100 μg/ml, gift from Y. Matsuda, Kyowa Hakko Kogyo) was used as a selective blocker of NPRs at concentrations previously shown to be effective in the isolated heart (30). To rule out the possibility that the effects of natriuretic peptides were mediated by the NO synthase-soluble guanylyl cyclase system, we repeated experiments in the presence of the soluble guanylyl cyclase inhibitor 1H-(1,2,4)-oxadiazolo-(4,3-a)-quinoxalin-1-one (ODQ, 10 μM, Tocris Cookson). This concentration abolishes the HR response to NO donors in a similar preparation (34). Milrinone (1 μM, Calbiochem) was used as a potent and selective inhibitor of PDE3, and PKA was inhibited using H-89 (0.5 μM, Calbiochem). These concentrations are above the reported inhibitory constant (K_i) for the isolated enzymes [0.3 μM milrinone for PDE3 (19), 0.05 μM H-89 for PKA (9)] but below those reported for nonspecific actions of the drugs. Presynaptic neuronal N-type calcium channels were blocked with ω-conotoxin GVIA (100 nM, Sigma) at concentrations that have previously been shown to be effective (28, 38). ω-Conotoxin has previously been shown to have no effect on the HR response to carbamylcholine (25).

The natriuretic peptides were dissolved in 5% acetic acid, and control experiments showed that only at the largest volume used (equivalent to 500 nM peptide) was bath pH significantly changed (pH 7.38 ± 0.01 to 7.31 ± 0.01 after 10 min, $n = 6$). However, this concentration had no effect on baseline HR or the response to vagal nerve stimulation at any frequency. Drugs were dissolved in reagent grade water

from an Elga purification system, except milrinone and H-89, which were dissolved in dimethylsulfoxide. A control experiment showed that dimethylsulfoxide at the concentrations used did not affect the HR response to vagal nerve stimulation at 1, 3, or 5 Hz. Because milrinone is light sensitive, these experiments were carried out in a darkened room.

Total RNA extraction and RT-PCR. The central region of the guinea pig sinoatrial node was dissected and immediately frozen in liquid nitrogen. Total RNA was isolated from the tissue after homogenization in an Eppendorf pestle and mortar (Anachem) using total RNA Extraction reagents (Ambion). RNA (1 μg) was reverse transcribed using the Expand Reverse Transcriptase System with oligo-dT_{15} (Roche Diagnostics). Oligonucleotide PCR primers were designed based on consensus sequences within published nucleotide sequences (GenBank database, NIH) for NPR type A (NPRA) (forward: 5′-AAG AGC CTG ATA ATC CTG AGT ACT-3′ and reverse: 5′-TTG CAG GCT GGG TCC TCA TTG TCA-3′), NPR type B (NPRB) (forward: 5′-AAC GGG CGC ATT GTG TAT ATC TGC GGC-3′ and reverse: 5′-TTA TCA CAG GAT GGG TCG TCC AAG TCA-3′), and β-actin (forward: 5′-TTC AAC TCC ATC ATG AAG AAG TGT GAC GTG-3′ and reverse: 5′-CTA AGT CAT AGT CCG CCT AGA AGC ATT-3′). Primers were synthesized commercially by MWG Biotech. Roche Diagnostics supplied PCR enzymes and buffers. Reactions contained 2 μl of cDNA from each reverse transcribed reaction, 1× phosphocreatine buffer, 1.5 mM $MgCl_2$, 200 μM 2-deoxynucleotide 5′-triphosphate mix, 20 pmoles of each primer, and 1.0 U *Taq* DNA polymerase in 50-μl volumes with nuclease-free H_2O. The cycling parameters were an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, primer extension at 72°C for 1 min, and by one final extension at 72°C for 5 min in an DNA Engine thermal cycler with heated lid (MJ Research). Twenty microliters from each reaction were then separated on a 1.5% Aquapor HR agarose (National Diagnostics) gel containing 500 μg/ml ethidium bromide in 40 mM Tris-acetate, 1 mM EDTA (TAE buffer) for 2 h at 40 V, and DNA was visualized under ultraviolet illumination.

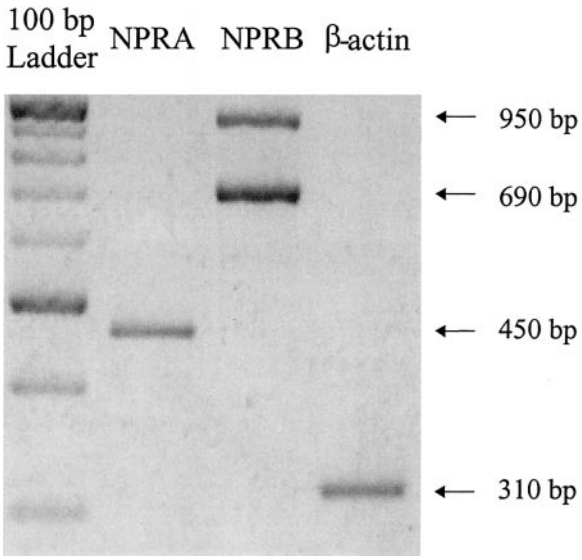

Fig. 1. Expression of natriuretic peptide receptor subtypes in guinea pig central sinoatrial node tissue. Electrophoresis gel showing the phosphocreatine (PCR) products (20 μl from a 50-μl reaction volume) for natriuretic peptide receptors type A (NPRA, 450 base pairs), type B (NPRB, 690 and 950 base pairs), and β-actin (positive control, 310 base pairs) from RNA isolated from central sinoatrial node tissue of a guinea pig. Equal amounts of cDNA from the same sinoatrial node were used in each reaction that underwent the same submaximal number of PCR cycles. Identical results were observed with central sinoatrial node tissue from another animal.

Table 1. *Natriuretic peptides directly stimulate heart rate independent of autonomic nervous system*

	Δ Baseline Heart Rate, beats/min				
	5 nM	10 nM	50 nM	100 nM	500 nM
ANP	−3 ± 3	−3 ± 5	6 ± 4	18 ± 4*	27 ± 3*
BNP	1 ± 1	6 ± 2	6 ± 4	11 ± 4*	26 ± 3*
CNP	12 ± 3*	20 ± 4*	25 ± 5*	28 ± 6*	39 ± 5*

Values are means ± SE. Atrial natriuretic peptide (ANP; $n = 6$), brain-derived natriuretic peptide (BNP; $n = 6$), and C-type natriuretic peptide (CNP; $n = 8$) significantly (*$P < 0.05$) increase baseline heart rate of isolated spontaneously beating guinea pig atria. Observed potency series of CNP ≫ BNP = ANP is consistent with the effect of a type-B natriuretic peptide receptor-mediated effect.

Statistical analysis. Data are presented as means ± SE. One-way repeated measures ANOVA, followed by Tukey's post hoc analysis was used to evaluate the effect of an intervention. An unpaired Student's *t*-test was used to evaluate the effect of an intervention between experimental groups. Statistical significance was accepted at $P < 0.05$.

RESULTS

Effect of natriuretic peptides on baseline HR. RT-PCR analysis of mRNA from tissue isolated from the central sinoatrial node region of the guinea pig heart showed the presence of NPRA and NPRB receptor products (Fig. 1). An identical result was observed in tissue isolated from another guinea pig.

In the spontaneously beating, double atrial preparation, mean baseline HR stabilized at 197 ± 2 beats/min ($n = 77$) after the equilibration period. All natriuretic peptides significantly increased baseline HR, although CNP ($n = 8$) was more potent than either ANP ($n = 6$) or BNP ($n = 6$) (see Table 1). This suggests that stimulation of the NPRB receptor is able to increase HR.

To investigate the mechanism for this tachycardia, the response to a single submaximal dose of CNP was evaluated in the presence of various inhibitors. The soluble guanylyl cyclase inhibitor ODQ had no effect on the HR response to CNP (ΔHR, 50 nM; CNP, +32 ± 8 beats/min; $n = 6$ vs. CNP + ODQ, +26 ± 1 beats/min; $n = 6$), but the specific NPR antagonist HS-142–1 significantly reduced the increase in HR to CNP ($n = 6$), suggesting that the tachycardia was due to stimulation of particulate guanylyl cyclase-coupled receptors. The response to CNP was also reduced by the PDE3 inhibitor milrinone ($n = 7$) and the blocker of I_f, cesium chloride ($n = 7$). However, cesium chloride may not completely block I_f at this concentration (2 mM) and may block potassium channels at higher concentrations (e.g., Refs. 14 and 32). We therefore repeated the experiment using the more specific I_f blocker ZD-7288 ($n = 6$), which produced similar effects (see Fig. 2).

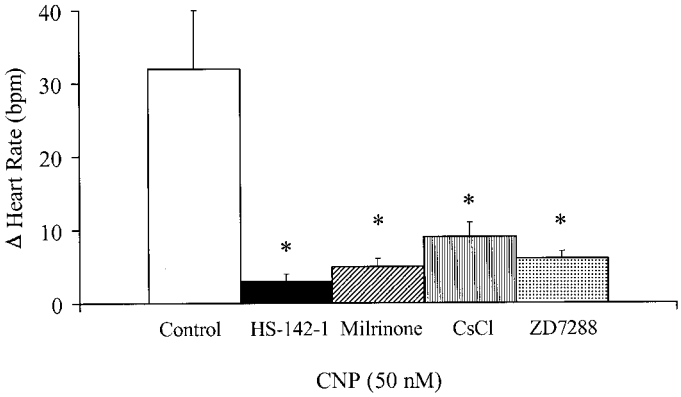

Fig. 2. Inhibitors of natriuretic peptide receptors, phosphodiesterase 3 and the hyperpolarization-activated current (I_f), prevent the tachycardia to C-type natriuretic peptide (CNP). Tachycardia to CNP (50 nM, $n = 7$) is significantly reduced (*$P < 0.05$, unpaired t-test) by natriuretic peptide receptor antagonist HS-142-1 (100 μg/ml, $n = 6$), phosphodiesterase 3 inhibitor milrinone (1 μM, $n = 7$), as well as the I_f blockers cesium chloride (2 mM, $n = 7$) and ZD-7288 (1 μM, $n = 6$).

Milrinone increased baseline HR by 62 ± 3 beats/min due to postsynaptic elevation of cAMP and stimulation of I_f (16) and cesium chloride, and ZD-7288 decreased baseline HR by 53 ± 7 and 95 ± 6 beats/min, respectively, as has been reported previously (31). When the response to CNP is expressed as a percent increase in HR, this did not affect statistical significance (ΔHR control, +18 ± 5%; HS-142–1, +1 ± 1%; milrinone, +2 ± 1%; cesium chloride, +7 ± 2%; ZD-7288, +7 ± 1%). This suggests that CNP acts via NPR to increase cGMP-dependent inhibition of PDE3 to raise cAMP levels. The increase in baseline HR to CNP may at least in part be due to stimulation of I_f.

Effect of natriuretic peptides on release of acetylcholine and HR response to vagal nerve stimulation and carbamylcholine. In addition to increasing baseline HR, CNP significantly increased the HR response to vagal nerve stimulation at all frequencies. BNP was less potent than CNP, and ANP had no significant effect on the vagal response at any concentration or frequency of stimulation (see Fig. 3).

To determine whether the action of CNP on the magnitude of vagal-induced bradycardia was independent of the concomitant increase in baseline HR, CNP (50 nM, $n = 7$) was administered in the presence of the I_f blocker cesium chloride. Cesium chloride significantly reduced the magnitude of the vagal-induced bradycardia (from −30 ± 2 to −25 ± 3 beats/min at 3 Hz and −55 ± 3 to −40 ± 4 beats/min at 5 Hz). However, as can be seen in Fig. 4, CNP was still able to augment the HR response to vagal nerve stimulation in the presence of cesium chloride, even though baseline HR does not significantly change.

CNP may increase the vagally mediated bradycardia by acting pre- or postsynaptically. We therefore compared the effects of a single dose of CNP (50 nM) on the HR response to vagal nerve stimulation to that mediated by the stable analog of acetylcholine, carbamylcholine. Figure 5 shows that CNP had no effect on the response to carbamylcholine at similar HRs to those observed during vagal stimulation. This supports the hypothesis that the action of CNP is independent of the change in baseline HR and suggests that CNP facilitates vagal neurotransmission by a presynaptic pathway.

To investigate whether CNP augments vagal neurotransmission by increasing the release of acetylcholine, we radioactively labeled right atrial acetylcholine stores and measured the increase in ^{3}H efflux following field stimulation. Control experiments showed that the increase in [^{3}H]acetylcholine release to two consecutive field stimulations remained constant over time (Fig. 6, *C* and *D*, $n = 4$). CNP (50 nM, $n = 6$), however, signifi-

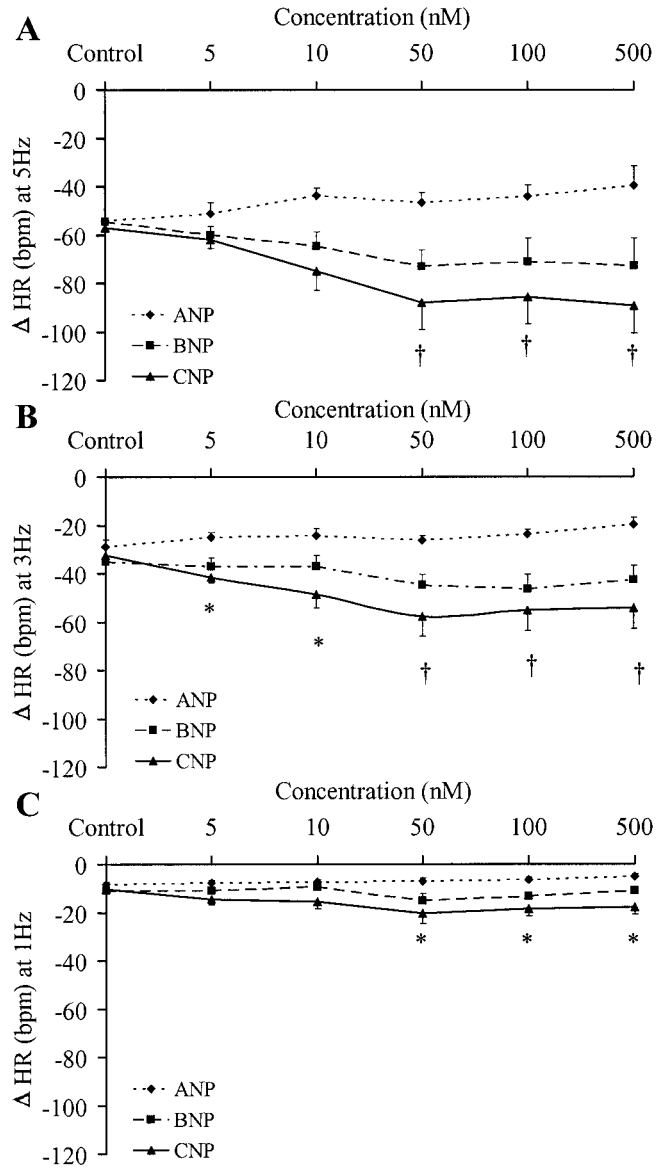

Fig. 3. Brain-derived natriuretic peptide (BNP) and CNP but not atrial natriuretic peptide (ANP) augment vagal bradycardia. Effects of natriuretic peptides (5–500 nM) on heart rate (HR) response (beats/min) to right vagal nerve stimulation at 5 (*A*), 3 (*B*), and 1 Hz (*C*). CNP ($n = 8$) and BNP ($n = 6$) significantly (*$P < 0.05$ for CNP, †$P < 0.05$ for BNP and CNP vs. control) increase the vagal bradycardia, whereas ANP ($n = 6$) has no significant effect at any frequency or concentration.

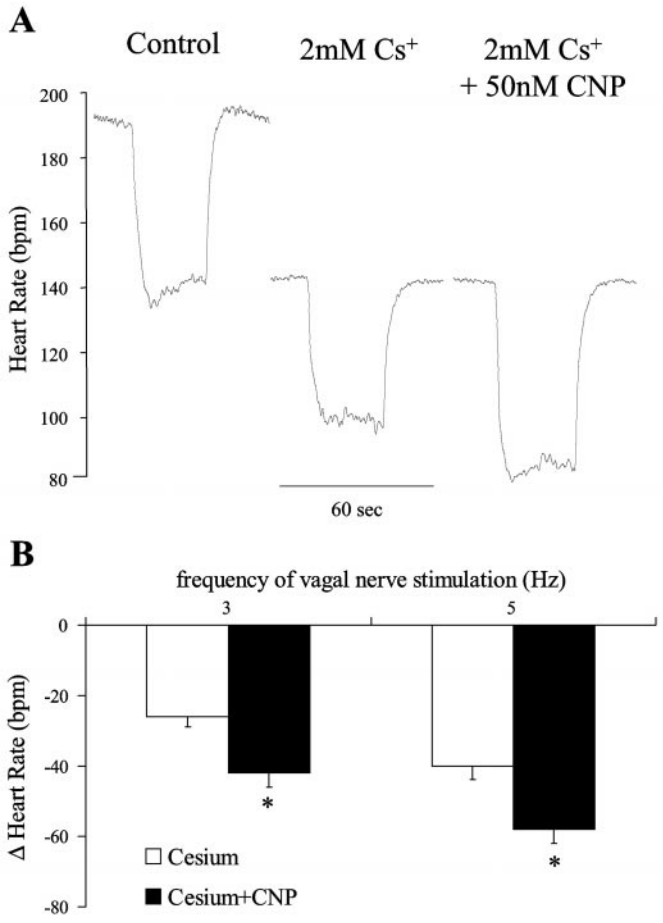

Fig. 4. CNP augments vagal bradycardia independent of increasing baseline HR. I_f blocker cesium chloride (2 mM, $n = 7$) decreases baseline HR and reduces magnitude of vagal bradycardia (*A*, raw data trace at 5 Hz). In presence of cesium chloride, CNP (50 nM) still significantly (*$P < 0.05$) increases HR response (beats/min) to right vagal nerve stimulation despite there being no change in baseline HR (*B*, mean data).

cantly increased the evoked release of [^{3}H]acetylcholine to field stimulation (Fig. 6, *A* and *B*).

Presynaptic pathway for the effect of CNP on the HR response to vagal nerve stimulation. Inhibition of PDE3 with milrinone significantly increased the magnitude of the vagal-induced bradycardia at 3- and 5-Hz stimulation frequencies (ΔHR at 3 Hz: −30 ± 2 control, −46 ± 7 milrinone, −31 ± 3 beats/min wash; at 5 Hz: −60 ± 2 control, −89 ± 6 milrinone, −59 ± 4 beats/min wash; $n = 7$). In keeping with this observation, the PKA inhibitor H89 reduced the magnitude of vagal-induced bradycardia (ΔHR at 3 Hz: −34 ± 2 control, −27 ± 1 beats/min H89; at 5 Hz: −64 ± 1 control, −49 ± 2 beats/min H89; $n = 6$). We have previously shown that milrinone and H89 have no effect on the HR response to carbamylcholine and that milrinone increases acetylcholine release (21). In the presence of either milrinone or H89, CNP has no effect on the HR response to vagal nerve stimulation (see Fig. 7).

Both P-type and N-type calcium channels are involved in cardiac vagal neurotransmission, and the effect of an NO donor can be abolished by inhibition of N-type channels only (21). After inhibition of N-type calcium channels had reduced the HR response to vagal nerve stimulation (ΔHR at 5 Hz: −62 ± 2 control, −14 ± 2 beats/min ω-conotoxin; at 7 Hz: −88 ± 4 control, −22 ± 5 beats/min ω-conotoxin; $n = 6$), CNP was also unable to have any effect (see Fig. 8, *A* and *B*). This suggests that CNP, like NO, acts via inhibition of PDE3 to increase cAMP-PKA-dependent phosphorylation of N-type calcium channels. The NPR receptor antagonist HS-142–1 also abolishes the effect of CNP (see Fig. 8, *C* and *D*). The lack of an effect of HS-142–1 on the response to vagal stimulation (ΔHR at 3 Hz: −35 ± 5 control, −30 ± 3 beats/min HS-142–1; at 5 Hz: −57 ± 2 control, −52 ± 1 beats/min HS-142–1; $n = 5$) suggests that in this organ bath preparation with constant preload, there is no endogenous release of natriuretic peptides. CNP still significantly increased the HR response to vagal nerve stimulation in the presence of the soluble guanylyl cyclase inhibitor ODQ (ΔHR at 5 Hz: −67 ± 1 control, −60 ± 2 ODQ, −75 ± 5 CNP + ODQ, −65 ± 6 beats/min wash; $n = 6$). This suggests that CNP is acting via particulate guanylyl cyclase-coupled receptors rather than through the NO synthase-soluble guanylyl cyclase system to raise intracellular cGMP.

DISCUSSION

The main new findings of this study are the following: *1*) natriuretic peptides can directly stimulate HR independent of the autonomic nervous system by a cGMP-dependent pathway that may involve a PDE3-dependent increase in cAMP and I_f; *2*) CNP and BNP increase the HR response to vagal nerve stimulation, and CNP acts via a presynaptic cGMP-dependent pathway that increases the release of acetylcholine; and *3*) CNP augments the vagal-induced bradycardia by cGMP stimulation of PDE3, increasing cAMP-PKA-dependent phosphorylation of presynaptic N-type calcium channels.

Natriuretic peptides can directly stimulate cardiac pacemaking. In the guinea pig sinoatrial node, we have shown that PCR fragments for both subtypes of NPRs are present and that natriureic peptides can directly stimulate HR. The effect of ANP and BNP on HR was confined to high concentrations of the peptides (100–500 nM) and may explain why others using lower concentrations have failed to observe any effect of ANP (5, 15, 22, 23). CNP is more potent and increases HR at relatively low concentrations, an effect abolished by the NPR antagonist HS-142–1 (5, 22). HS-142–1 is a specific antagonist for type A and B particulate guanylyl cyclase-coupled receptors (27), and therefore, it is unlikely that stimulation of the type C NPR that can be positively coupled to cAMP production via adenylyl cyclase (45) is responsible for the effects of CNP. The soluble guanylyl cyclase inhibitor ODQ has no effect on the HR response to CNP, suggesting the tachycardia is not due to stimulation of the NO synthase-soluble guanylyl cyclase system. These results are consistent with the hypothesis that the tachycardia is mediated

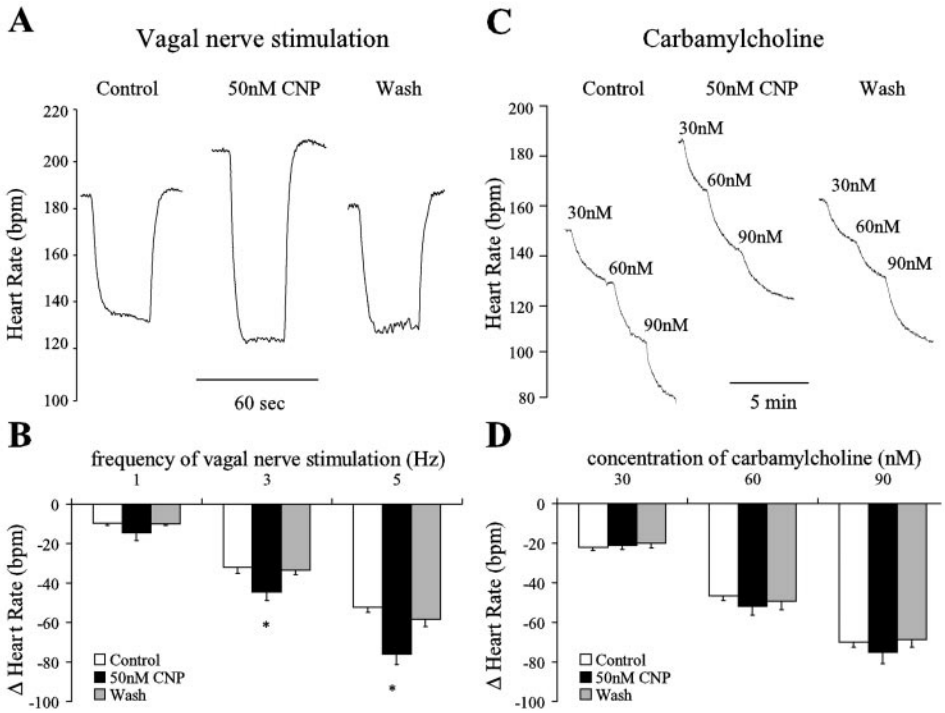

Fig. 5. CNP augments cholinergic-induced bradycardia via a presynaptic mechanism. CNP (50 nM, $n = 6$) significantly increases (*$P < 0.05$) HR response (beats/min) to right vagal nerve stimulation, and this effect was reversed with wash out of the drug (*A*, raw data trace at 5 Hz, and *B*, mean data). However, CNP had no effect on HR response to carbamylcholine ($n = 8$) (*C* and *D*), suggesting that CNP augments vagal bradycardia via a presynaptic mechanism.

by a type B particulate guanylyl cyclase-coupled NPR for which CNP is the natural ligand (46). Importantly, the type B NPR is more potent at increasing intracellular cGMP than the type A receptor (18, 41).

Of interest, NO donors can also act directly on the heart to cause tachycardia (24, 34). This is mediated by an increase in the rate of diastolic depolarization and magnitude of I_f (34) and is at least in part due to cGMP-dependent inhibition of PDE3 and increasing levels of cAMP (35). cAMP acts directly on the hyperpolarization-activated cyclic-nucleotide-gated channel channel to shift its activation curve to more positive potentials (e.g., Ref. 16). The tachycardia to CNP is also due to an increase in the rate of diastolic depolarization (5), and we have shown that inhibition of PDE3 and blockers of I_f significantly reduce the HR response to CNP. This suggests that NO and CNP may share a common cGMP-dependent mechanism to increase HR, although direct measurements of CNP on I_f are needed to confirm this hypothesis.

BNP and CNP augment vagal neurotransmission. Whereas BNP and CNP augment the HR response to vagal nerve stimulation at physiological frequencies, ANP had no effect at any concentration or frequency of

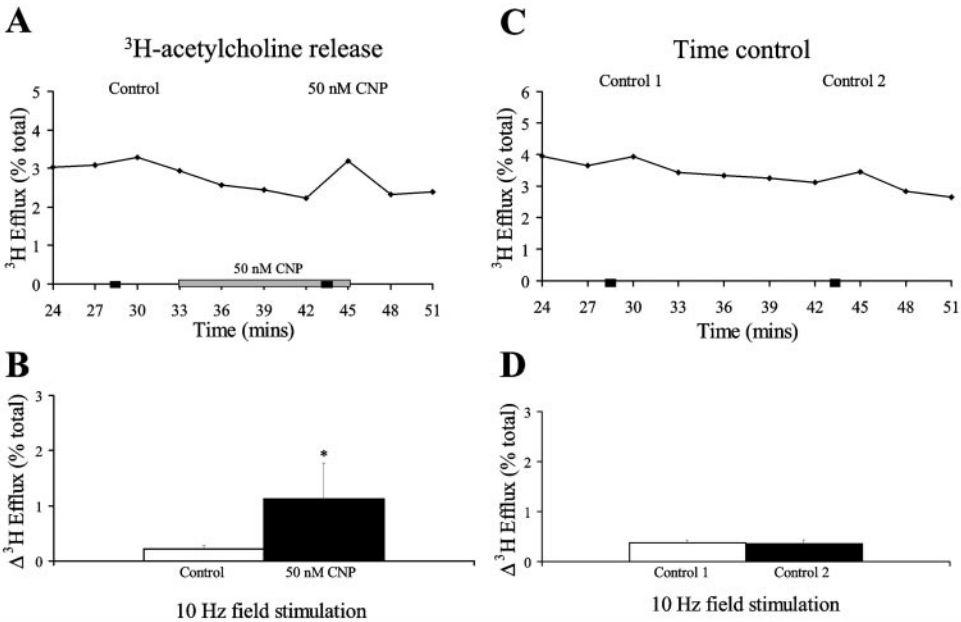

Fig. 6. CNP increases [^{3}H]acetylcholine release to field stimulation. CNP (50 nM, $n = 6$) significantly increases (*$P < 0.05$) ^{3}H efflux (sampled every 3 min) to field stimulation (10 Hz, at 30 and 45 min) (*A* and *B*). Control experiments ($n = 4$) showed that the increase in ^{3}H efflux to field stimulation remained constant over time (*C* and *D*).

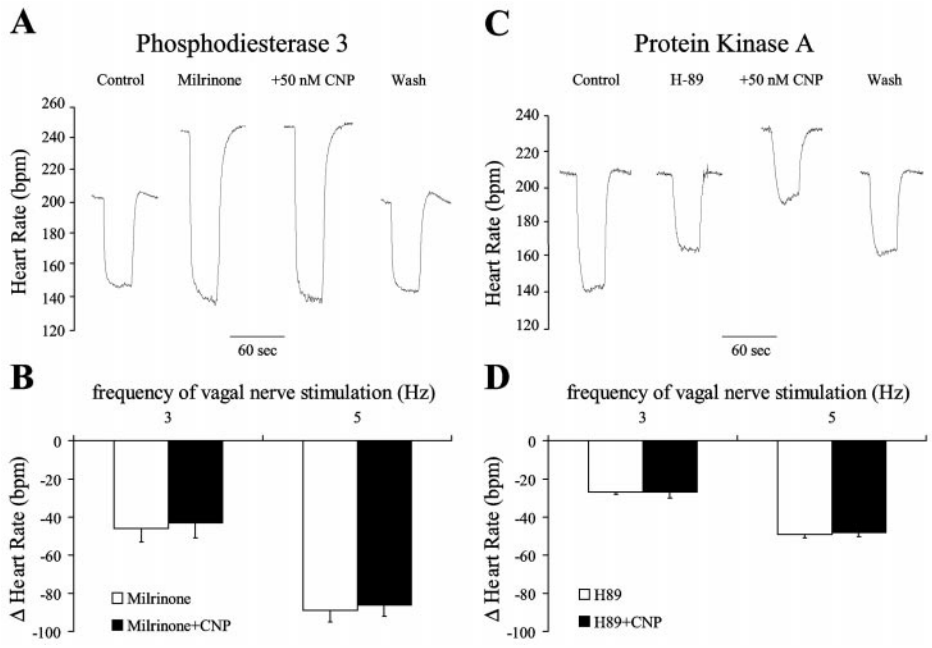

Fig. 7. CNP augments vagal bradycardia via a mechanism involving phosphodiesterase 3 and protein kinase A. Inhibition of phosphodiesterase 3 (1 μM milrinone, $n = 7$) significantly increases the magnitude of vagal bradycardia as we have previously reported. However, CNP (50 nM) has no effect on the HR response (beats/min) to right vagal nerve stimulation in the presence of phosphodiesterase 3 inhibition (*A*, raw data at 5 Hz, and *B*, mean data). In keeping with this result, inhibition of protein kinase A (0.5 μM H-89, $n = 6$) significantly reduces the vagal bradycardia, but CNP has no effect on the response to vagal nerve stimulation in the presence of protein kinase A inhibition (*C*, raw data at 5 Hz, and *D*, mean data).

stimulation. Because all natriuretic peptides increase baseline HR, this suggests that the increase in the magnitude of the vagal-induced bradycardia is specific to the peptide used rather than being dependent on the shift in baseline HR. This was confirmed by the observation that CNP still augmented the vagal-induced bradycardia when baseline HR was held constant with the I_f blocker cesium chloride. Whereas the lack of an effect of ANP agrees with some studies (1, 23), others have shown that ANP augments the bradycardia to efferent vagal nerve stimulation in vivo (2). This may be due to an interaction with adrenergic-cholinergic cross talk via α_1-adrenergic receptors on parasympathetic ganglia because the effect of ANP on vagal nerve stimulation can be blocked by prazosin (3). However, it is not clear whether ANP is modulating the response to α_1-receptor stimulation or whether the effect is secondary to an action of ANP on the release of catecholamines. Whereas cross talk of this nature may occur in vivo, circulating catecholamines and norepinephrine release is lacking in the in vitro preparation used for this study.

The potency series of CNP > BNP for their action on vagal neurotransmission and the ability of HS-142–1, but not ODQ, to prevent the effect of CNP suggests that the response is mediated by particulate guanylyl

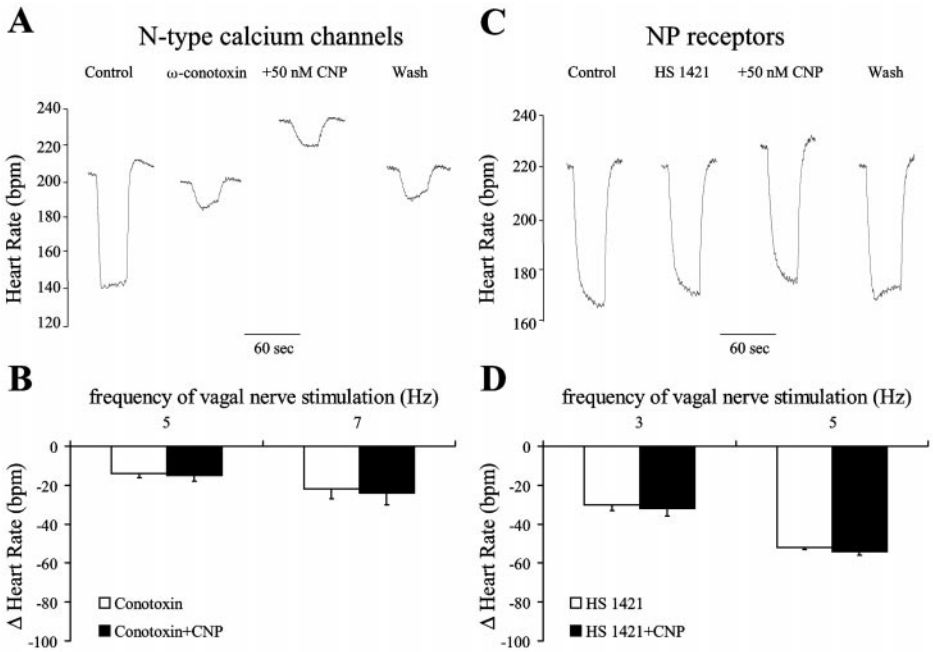

Fig. 8. CNP augments vagal bradycardia via a natriuretic peptide receptor-mediated pathway involving N-type calcium channels. Blocking N-type calcium channels (100 nM ω-conotoxin, $n = 6$) significantly reduces but does not abolish HR response (beats/min) to right vagal nerve stimulation as we have previously reported. However, CNP (50 nM) has no effect on the magnitude of the vagal bradycardia after block of N-type calcium channels (*A*, raw data at 5 Hz and *B*, mean data). Antagonizing natriuretic peptide receptors (HS-142-1, 100 μg/ml, $n = 5$) has no efect on the vagal bradycardia but prevents any further effect of CNP (50 nM) (*C*, raw data at 5 Hz and *D*, mean data).

cyclase-coupled NPRB. In atrial tissue CNP and BNP are more potent than ANP at stimulating cGMP production (30). The lack of an effect of CNP on the HR response to carbamylcholine suggests that the site of action of this peptide is presynaptic. The membrane-permeable analog of cGMP 8-bromo-cGMP augments vagal-induced bradycardia via a presynaptic mechanism (43), as can NO donors via stimulating soluble guanylyl cyclase (21). In addition, we show for the first time that CNP facilitates the release of [^{3}H]labeled acetylcholine during field stimulation in isolated atria, an effect also shared by NO donors (21).

Presynaptic pathway for cGMP in vagal control of HR. Inhibition of PDE3 augments the release of acetylcholine to increase the HR response to vagal nerve stimulation (21). Given that CNP also acts to increase cGMP and produce similar effects to NO, it is not surprising that its action can also be prevented by PDE3 inhibition. The effect of NO donors on the magnitude of the vagal-induced bradycardia is not affected by inhibition of PDE2 or protein kinase G but can be prevented by several inhibitors of PKA and blockers of the N-type calcium channels (21). Similarly, we show the effect of CNP was prevented by inhibition of PKA and N-type calcium channels despite the postsynaptic stimulation of pacemaking remaining intact. Others have also shown that the presynaptic cAMP-PKA system can modulate cardiac vagal neurotransmission. Both the membrane permeable cAMP analog 8-bromo-cAMP (13) and stimulation of adenylate cyclase with pituitary adenylate cyclase-activating polypeptide (44) increases the release of radioactively labeled acetylcholine in isolated atria. Given that both BNP and CNP increase the HR response to vagal nerve stimulation and both peptides act on similar guanylyl cyclase-coupled natriuretic peptide receptors, it would be reasonable to assume that the action of BNP is mediated via the same intracellular pathway as CNP to facilitate acetylcholine release (Fig. 9).

Functional implications. In the beat-to-beat regulation of cardiac output, an increase in venous return to the heart produces an increase in both stroke volume (the Frank-Starling effect) and HR (the Bainbridge effect). The Bainbridge effect is mediated in part by reflex vagal withdrawal, but also by intrinsic cardiac mechanisms because the phenomenon can be observed in the isolated heart (6). This may be due to opening of stretch-sensitive ion channels in sinoatrial node cells (12), although an increase in myocardial stretch also increases the release of ANP that we have shown is capable of stimulating the heart directly. Whether this paracrine modulation of HR occurs in vivo is not clear, from our dose-response curves, local concentrations of at least 100 nM would be required for such an effect. We also observed no effect of ANP on the HR response to vagal nerve stimulation. In keeping with this observation, a transient increase in right atrial pressure does not augment the HR response to vagal nerve stimulation (7).

Although plasma concentrations of BNP and CNP are very low (39), during conditions of chronic volume overload and persistent cardiac stretch, the expression of BNP is greatly increased in both the atria and the ventricles (33). Plasma levels of BNP during severe congestive heart failure rise by up to 30-fold (36), while plasma levels of CNP remain unchanged (48). It is well established that cholinergic activation antagonizes the potentially prodysrhythmic effects of high sympathetic tone on the heart (29), and high vagal tone is a good prognostic indicator against sudden cardiac death (11). An effect of BNP on vagal neurotransmission in these conditions could conceivably represent a compensatory protective mechanism for the prevention of adrenergic-induced dysrhythmia.

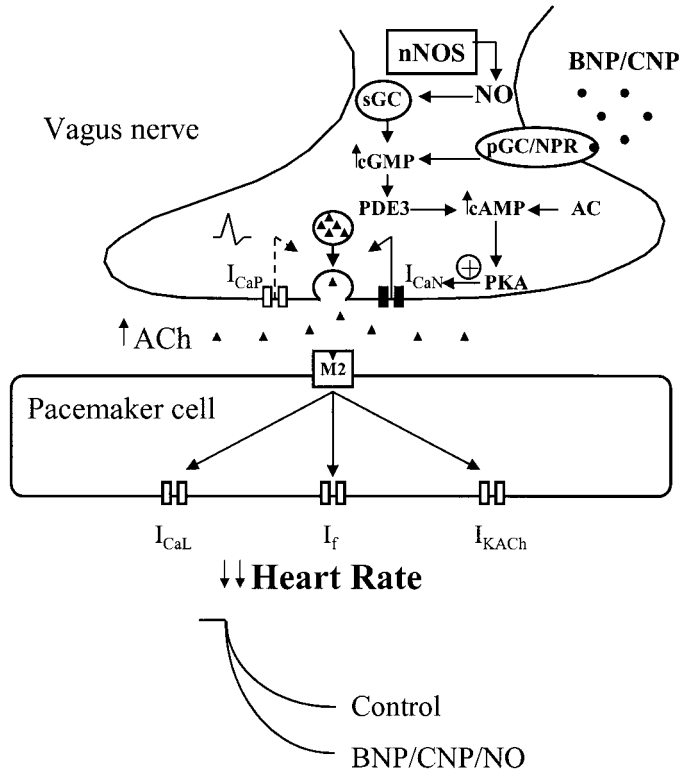

Fig. 9. Nitric oxide (NO) and natriuretic peptides facilitate vagal bradycardia via a cGMP-dependent pathyway. Stimulation of the vagus nerve causes opening of N-type (I_{CaN}) and P-type (I_{CaP}) voltage-gated calcium channels, influx of calcium, and the exocytotic release of acetylcholine (ACh). ACh acts on postsynaptic muscarinic (M2) receptors on sinoatrial node pacemaker cells to decrease HR via changes in several pacemaking currents such as L-type calcium current (I_{CaL}), I_f, and ACh-sensitive potassium current (I_{KACh}). NO produced by neuronal NO synthase (nNOS) facilitates the release of ACh via stimulation of presynaptic soluble guanylyl cyclase (sGC) and an increase in cGMP. Natriuretic peptides BNP and CNP can also increase presynaptic cGMP and produce similar effects via stimulation of particulate guanylyl cyclase-coupled natriuretic peptide receptors (pGC/NPR). cGMP inhibits phosphodiesterase 3 to increase cAMP-protein kinase A-dependent phosphorylation of N-type calcium channels. This pathway may augment the HR response to vagal nerve stimulation by increasing presynaptic calcium influx and vesicular release of acetylcholine.

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