An Unsuspected Property of Natriuretic Peptides
Promotion of Calcium-Dependent Catecholamine Release Via Protein Kinase G-Mediated Phosphodiesterase Type 3 Inhibition

Noel Yan-Ki Chan, BS; Nahid Seyedi, PhD; Kenichi Takano, MD, PhD; Roberto Levi, MD, DSc

Background—Although natriuretic peptides are considered cardioprotective, clinical heart failure trials with recombinant brain natriuretic peptide (nesiritide) failed to prove it. Unsuspected proadrenergic effects might oppose the anticipated benefits of natriuretic peptides.

Methods and Results—We investigated whether natriuretic peptides induce catecholamine release in isolated hearts, sympathetic nerve endings (cardiac synaptosomes), and PC12 cells bearing a sympathetic neuron phenotype. Perfusion of isolated guinea pig hearts with brain natriuretic peptide elicited a 3-fold increase in norepinephrine release, which doubled in ischemia/reperfusion conditions. Brain natriuretic peptide and atrial natriuretic peptide also released norepinephrine from cardiac synaptosomes and dopamine from nerve growth factor–differentiated PC12 cells in a concentration-dependent manner. These catecholamine-releasing effects were associated with an increase in intracellular calcium and abolished by blockade of calcium channels and calcium transients, demonstrating a calcium-dependent exocytotic process. Activation of the guanylyl cyclase-cyclic GMP-protein-kinase-G system with nitroprusside or membrane-permeant cyclic GMP analogs mimicked the proexocytotic effect of natriuretic peptides, an action associated with an increase in intracellular cyclic AMP (cAMP) and protein-kinase-A activity. Cyclic AMP enhancement resulted from an inhibition of phosphodiesterase type 3–induced cAMP hydrolysis. Collectively, these findings indicate that, by inhibiting phosphodiesterase type 3, natriuretic peptides sequentially enhance intracellular cAMP levels, protein kinase A activity, intracellular calcium, and catecholamine exocytosis.

Conclusions—Our results show that natriuretic peptides, at concentrations likely to be reached at cardiac sympathetic nerve endings in advanced congestive heart failure, promote norepinephrine release via a protein kinase G–induced inhibition of phosphodiesterase type 3–mediated cAMP hydrolysis. We propose that this proadrenergic action may counteract the beneficial cardiac and hemodynamic effects of natriuretic peptides and thus explain the ineffectiveness of nesiritide as a cardiac failure medication.

Key Words: catecholamines ■ heart failure ■ natriuretic peptides

Plasma levels of brain natriuretic peptide (BNP) and norepinephrine (NE) increase with the severity of congestive heart failure (CHF). In fact, BNP and NE are regarded as important biomarkers for the diagnosis, risk stratification, and prediction of death in patients with CHF.1,2 Natriuretic peptides are generally viewed as cardioprotective because they were found to reduce blood pressure, plasma volume,3,4 and myocardial infarct size.5 Yet, other investigators reported an enlargement of infarct size after atrial natriuretic peptide (ANP) administration6 and a reduction in infarct size when the natriuretic peptide type A receptor was abrogated or blocked7 or when ANP was deleted.8 Moreover, although natriuretic peptides are thought to retard the progression of heart failure,9 the administration of recombinant BNP (nesiritide) was recently found to lack protective efficacy in acute heart failure patients.9 Thus, even though natriuretic peptides have been viewed as a compensatory neurohormonal system that is upregulated in the setting of heart failure, affording beneficial cardiac and hemodynamic effects,8 their role in alleviating cardiac ailments remains unsettled.

Clinical Perspective on p 307

The beneficial actions of natriuretic peptides are attributed to the stimulation of particulate guanylyl cyclase, and thus to an increased formation of cyclic GMP (cGMP),10 which has smooth muscle–relaxing and vasodilating effects,11 hypothetically related to decreased NE release from adrenergic nerves.12 A BNP-induced facilitation of cardiac vagal neurotransmission and bradycardia has also been reported.13 Nonetheless, injection of a membrane-permeable cGMP analog was found to increase heart rate before any changes in blood pressure,14 possibly because of enhanced NE release from sympathetic nerve endings. Indeed, the nitric oxide-
guanylyl cyclase-cGMP system has been shown to promote NE outflow in the mesenteric vasculature. Whether cGMP has sympatholytic16–18 or proadrenergic14,19–21 effects remains controversial. Thus, it is not inconceivable that an increase in intracellular levels of cGMP mediated by natriuretic peptides could actually contribute to the heightened cardiac sympathetic drive and excessive NE release that characterize CHF and atrial fibrillation.22,23

Given that an enhanced NE release bears dysfunctional and arrhythmogenic consequences,24–26 we investigated the NE-releasing properties of natriuretic peptides and cGMP. We report that BNP increases NE release in the guinea pig heart ex vivo, an effect that is further enhanced in ischemia/reperfusion. Furthermore, natriuretic peptides, sodium nitroprusside (SNP) and cell-permeable cGMP analogs all elicit catecholamine exocytosis in sympathetic nerves isolated from the guinea pig heart (ie, cardiac synaptosomes) and in nerve growth factor (NGF)–differentiated PC12 cells, which bear a sympathetic nerve-ending phenotype. This proexocytotic effect likely results from an increase in intracellular calcium (Ca2+). The process involves a protein kinase G (PKG)–mediated inhibition of phosphodiesterase type 3 (PDE3), which increases cAMP and protein kinase A (PKA) activity.

Methods

Ex Vivo Hearts

Twenty-one male Hartley guinea pigs (300–350 g; Charles River Laboratories, Kingston, NY) were anesthetized with CO2 and euthanized by stunning with approval from the Institutional Animal Care and Use Committee. Isolated hearts were perfused at constant pressure (55 cm H2O) with oxygenated (5% CO2, 95% O2) Ringer at 37°C in a Langendorff apparatus (Radnoti Glass Technology, Monrovia, CA). After equilibration, a number of hearts were perfused with BNP for 6 minutes. Other hearts were subjected to 10 minutes of global ischemia followed by 10 minutes of reperfusion in the absence or presence of BNP. Coronary flow was measured every 2 minutes; samples were assayed for NE. Surface ECG was obtained from the left ventricle and right atrium, recorded in digital format, and analyzed with Power Laboratory/8SP (AdInstrument, Colorado Springs, CO).

Preparation of Cardiac Synaptosomes

Thirty-seven guinea pig hearts were isolated as described in the above section. Hearts were perfused at constant pressure in a Langendorff apparatus with oxygenated Ringer solution for 15 minutes to ensure that no blood traces remained in the coronary circulation.27 Hearts were subsequently freed from fat and connective tissue and minced in ice-cold 0.32 mol/L sucrose containing 1 mM ethylenediaminetetraacetic acid tetrakis (acetoxymethyl ester) (10 μmol/L), the intracellular Ca2+ chelator BAPTA-AM [1,2-Bis(2-aminophenoxy)ethane-N,N,N’-tetraacetic acid tetrakis (acetoxyethyl ester)] (10 μmol/L), the cell-permeable protein kinase inhibitor PKI14–22 amide myristoylated (20 μmol/L), Rp-8-Br-cGMP (0.5 μmol/L), insulin (100 μmol/L), or cilostamide (10 μmol/L; 10 minutes). When these drugs were used, PC12 cells were preincubated with them for 10 minutes. Controls were incubated for an equivalent length of time without drugs. At the end of each experiment, aliquots of the supernatant and cell lysates (after a 30-minute treatment with Triton X-100) were taken from each well and analyzed for dopamine (DA) content by high-performance liquid chromatography-EC with a 6-minute retention time.28

Intracellular cAMP and cGMP Assay

PC12 cells were washed twice with Na Ringer and then treated with potassium (100 mmol/L; 3 minutes) or BNP (100 mmol/L; 10 minutes) in the presence or absence of RP-8-Br-cGMP (0.5 μmol/L; 10 minutes), insulin (100 μmol/L; 10 minutes), or cilostamide (10 μmol/L; 10 minutes). Controls were incubated for an equivalent length of time without drugs. At the end of each experiment, cells were washed twice with Dulbecco phosphate-buffered saline containing 10 mmol/L EGTA (to chelate external Ca2+) and then with normal phosphate-buffered saline to remove the remaining EGTA. Cells were then lysed with addition of water and harvested with a scraper. The Ca2+ content was determined using a Ca2+ assay kit (Quantichrom Ca2+ Assay Kit by BioAssay Systems, Hayward, CA). The Ca2+ content was adjusted by the protein content of the cells and expressed as milligrams of Ca2+/milligram of protein.

cAMP Assay

PC12 cells were treated and lysed as described above. Intracellular cAMP levels were determined using a cAMP Biotrak EIA kit (GE Healthcare Biosciences Corp, Piscataway, NJ) following the manufacturer’s protocol.28 This cAMP assay is highly specific and is based on competition between unlabeled cAMP and a fixed quantity of peroxidase-labeled cAMP for a limited number of binding sites on a cAMP-specific antibody. The cross-reactivity for cGMP, AMP, ADP, and ATP is <0.01% whereas cAMP is 100%.

PKA Activity

Protein kinase A phosphorylation (ie, an indication of PKA activation) was measured using a p-PKAα antibody (Santa Cruz Biotech-
nology Inc, Santa Cruz, CA) in Western blot. Methods for Western blot analysis were as previously described.27

PKG Activity
Phosphorylated vasodilator-stimulated phosphoprotein (VASP, a major substrate for PKG) at Ser239 is a sensitive biochemical marker for monitoring the activity of PKG.31 Vasodilator-stimulated phosphoprotein phosphorylation (ie, PKG activity) was measured using a p-VASP antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) in Western blot. Methods for Western blot analysis were as previously described.27

PDE3 Activity
Phosphodiesterase type 3 activity was measured using a commercially available colorimetric PDE assay kit (Biomol International Inc, Plymouth Meeting, PA) as previously described.32 PC12 cell lysates were prepared, and then total protein concentration was measured as described above. Free phosphate contamination was removed according to manufacturer’s protocol. Samples were incubated for 10 minutes at 37°C, and reactions were stopped with a major substrate for PKG (Ser239) phosphorylation.31 Methods for Western blot analysis were as previously described.27

Drugs and Chemicals
Brain natriuretic peptide and ANP were purchased from AnaSpec (Fremont, CA); SNP, ω-CTX, nifedipine, 8-bromo-cGMP, 8-pCPT-cGMP, forskolin, PKI14–22, Rp-8-Br-cGMPS, LY-83583, insulin, and cilostamide were purchased from Sigma-Aldrich (St Louis, MO); BAPTA-AM was purchased from Invitrogen (Carlsbad, CA).

Statistics
Data are presented as mean±SEM. Parametric tests were used throughout the study. For the type of experiment involving repeated measures over time, repeated measures ANOVA was performed for statistical analysis, followed by posthoc pairwise comparisons of the time points. The Dunnett multiple comparison test was used to adjust the posthoc pairwise comparisons of the time points (Figure 1A). For 2-group comparisons, the unpaired t test was used in all the other figures. In addition, to confirm the significance of the parametric findings, we have calculated P values using nonparametric tests (ie, Mann-Whitney test and Kruskal-Wallis test followed by the posthoc Dunn test). GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA) was used. Values of P<0.05 were considered statistically significant.

Results
Myocardial Ischemia/Reperfusion Enhances the NE-Releasing Effects of BNP
Continuous administration of BNP (100 nmol/L) to Langendorff-perfused guinea pig hearts ex vivo caused a ~2-fold increase in NE overflow, which reached a peak within 2 to 4 minutes and persisted until BNP administration was discontinued (Figure 1A). Notably, in hearts undergoing 10-minute global ischemia, NE release increased 3 fold in the first 2 minutes of reperfusion, and the addition of BNP (100 nmol/L) caused a further ~2-fold increase in the same 2-minute period (Figure 1B). Hence, the NE-releasing effects of BNP are enhanced in the setting of ischemia/reperfusion.

Guanylyl Cyclase Stimulation Induces an Increase in Catecholamine Release
We next evaluated this NE-releasing effect at the level of sympathetic nerve terminals (ie, cardiac synaptosomes). In a concentration-dependent manner, BNP and ANP each elicited an ~12% to 35% increase in NE release from cardiac synaptosomes, (>EC50 ≅20 nmol/L; Figure 2A, a and b). Similarly, in a concentration-dependent manner, BNP and ANP each elicited an ~10% to 50% increase in DA release (EC50 ≅5 nmol/L; Figure 2B, a and b) from NGF-differentiated PC12 rat pheochromocytoma cells, a cellular model of sympathetic neuron.33

Because BNP and ANP are both activators of particulate guanylyl cyclase,4 we next assessed whether activation of soluble guanylyl cyclase would also increase catecholamine release. We found that incubation with SNP (3–100 nmol/L; a soluble guanylyl cyclase activator) induced an ~6% to 28% increase in NE from cardiac synaptosomes (EC50 ≅20 nmol/L; Figure 2A, a and b) and an ~5% to 25% increase in DA release from PC12 cells (EC50 ≅5 nmol/L; Figure 2B, c). Collectively, these results suggested that stimulation of guanylyl cyclase induces an increase in catecholamine release.

The Catecholamine-Releasing Effect of cGMP Is Ca2+ Dependent
Because guanylyl cyclase activators elicited catecholamine release from sympathetic nerve endings (Figures 1 and 2), we next assessed whether cGMP would also promote NE and DA release. We found that, as a function of its concentration, the membrane-permeable analog of cGMP, 8-bromo-cGMP (0.1–100 μmol/L), induced an increase in the release of NE and DA from cardiac synaptosomes (10% to 35%; ED50 ≅10 μmol/L) and PC12 cells (25% to 80%; ED50 ≅3 μmol/L), respectively (Figure 3 A–D). In the presence of the N-type Ca2+ channel inhibitor ω-CTX GV1a (100 nmol/L)34 or the L-type Ca2+ channel inhibitor nifedipine (5 μmol/L),35 the concentration-response curve for the NE-releasing effects of 8-bromo-cGMP was mark-
PKG inhibition or PDE3 stimulation (Figure 3E). This strengthened the findings, with Ca^{2+} channel inhibitors and chelator indicating that an increase in intracellular Ca^{2+} mediates the promotion of catecholamine release by natriuretic peptides and cGMP analogs.

**Catecholamine-Releasing Effect of BNP and cGMP Relies on the Activation of the cAMP-PKA Pathway**

The administration of BNP (100 nmol/L), and the 2 membrane-permeable cGMP analogs 8-bromo-cGMP (1 μmol/L) and 8-pCPT-cGMP (3 μmol/L) to PC12 cells induced in each case an ≈2-fold increase in intracellular cAMP level whereas the adenylyl cyclase activator forskolin (10 μmol/L) elicited an ≈30-fold increase in intracellular cAMP (Figure 4A). Comparably, the administration of BNP (100 nmol/L), 8-bromo-cGMP (1 μmol/L), 8-pCPT-cGMP (3 μmol/L), and forskolin (10 μmol/L) to PC12 cells induced in each case an ≈90% increase in PKA activity (Figure 4B). Notably, the 8-bromo-cGMP–induced increase in neurotransmitter release in cardiac synaptosomes and PC12 cells was markedly attenuated by PKA inhibition with PKI_{14–22} (20 nmol/L) (Figure 4C). Collectively, these data indicated that BNP- and cGMP-mediated enhancements of catecholamine exocytosis rely on an increase in PKA activity because of an increase in intracellular cAMP.

**Catecholamine-Releasing Effect of BNP and cGMP Relies on a PKG-Induced Activation of the cAMP-PKA Pathway**

Our results suggested that an activation of the cAMP-PKA pathway plays a role in the catecholamine-releasing effect of BNP and cGMP (Figure 4). Because natriuretic peptides are known to signal via PKG,

3,4 we next investigated whether PKG activation might in turn activate the cAMP-PKA pathway plays a role in the catecholamine-releasing effect of BNP and cGMP. Because natriuretic peptides are known to signal via PKG,

3,4 we next investigated whether PKG activation might in turn activate the cAMP-PKA pathway plays a role in the catecholamine-releasing effect of BNP and cGMP. Because natriuretic peptides are known to signal via PKG,

3,4 we next investigated whether PKG activation might in turn activate the cAMP-PKA pathway plays a role in the catecholamine-releasing effect of BNP and cGMP. Because natriuretic peptides are known to signal via PKG,

3,4 we next investigated whether PKG activation might in turn activate the cAMP-PKA pathway plays a role in the catecholamine-releasing effect of BNP and cGMP. Because natriuretic peptides are known to signal via PKG,

3,4 we next investigated whether PKG activation might in turn activate the cAMP-PKA pathway plays a role in the catecholamine-releasing effect of BNP and cGMP. Because natriuretic peptides are known to signal via PKG,

3,4 we next investigated whether PKG activation might in turn activate the cAMP-PKA pathway plays a role in the catecholamine-releasing effect of BNP and cGMP. Because natriuretic peptides are known to signal via PKG,

3,4 we next investigated whether PKG activation might in turn activate the cAMP-PKA pathway plays a role in the catecholamine-releasing effect of BNP and cGMP. Because natriuretic peptides are known to signal via PKG,
that the BNP-induced promotion of catecholamine exocytosis involves an increase in PKG activity followed by a rise in intracellular cAMP and augmented PKA activity.

**Catecholamine-Releasing Effect of BNP Depends on an Increase in Intracellular cAMP Resulting From an Inhibition of PDE3-Induced cAMP Hydrolysis**

Because cGMP is known to inhibit PDE3,4 we next investigated whether the cAMP-dependent proexocytotic effects of BNP may result from PDE3 inhibition. We first used insulin and cilostamide, known to stimulate and inhibit PDE3, respectively.40,41 We found that insulin (30 nmol/L and 100 nmol/L) markedly inhibited the increase in NE and DA release induced by BNP (100 nmol/L) in cardiac synaptosomes and PC12 cells, respectively, and that the effect of insulin was reversed by cilostamide (10 μmol/L) (Figure 6A).

As these findings indirectly suggested a BNP-mediated inhibition of PDE3, we next measured PDE3 activity in PC12 cell lysate and its response to BNP. As expected,40,41 insulin (100 nmol/L) stimulated PDE3 activity by ~60% whereas cilostamide (10 μmol/L) inhibited it by ~30% (Figure 6C). Like cilostamide, BNP (100 nmol/L) also inhibited PDE3 activity by ~30%, an effect that was prevented by the PKG inhibitor Rp-8-Br-cGMPs (0.5 μmol/L) (Figure 6C). Stimulation of PDE3 with insulin (100 nmol/L) prevented the inhibitory effect of BNP, an effect that was abolished by cilostamide (10 μmol/L) (Figure 6C). These findings suggested that BNP inhibits PDE3 in a PKG-dependent manner.

Having demonstrated that BNP inhibits PDE3 activity, we next assessed the effect of this action on cellular cAMP level.

We found that BNP (100 nmol/L) enhanced cAMP level in PC12 cells by ~120% (Figure 6B). Phosphodiesterase type 3 stimulation with insulin (100 nmol/L) prevented the BNP-induced increase in cAMP, an effect that was reversed by inhibition of PDE3 with cilostamide (10 μmol/L) (Figure 6B). Similarly, BNP (100 nmol/L) enhanced PKA activation by ~3 fold and this effect was prevented by insulin (100 nmol/L) but restored by cilostamide (10 μmol/L) (Figure 6D). Notably, the BNP-induced increase in PKA activity was similar to that elicited by forskolin (10 μmol/L), used here as a positive control (Figure 6D). Collectively, these findings indicated that, by inhibiting PDE3, BNP sequentially enhances intracellular cAMP levels, PKA activity, Ca\(^{2+}\) content, and catecholamine exocytosis.

**Discussion**

The purpose of this study was to characterize a novel and unsuspected property of natriuretic peptides; i.e., the promotion of NE exocytosis. We report that not only natriuretic peptides but also SNP and cell-permeable cGMP analogs all elicit catecholamine exocytosis. This effect is mediated by an increase in intracellular Ca\(^{2+}\) and associated with an increase in intracellular cAMP and PKA activity resulting from a PKG-mediated inhibition of PDE3 (Figure 7).

What prompted our investigation was the previous experimental evidence that ANP administration increases infarct size and mortality in mice with coronary ligation6 and the recent clinical report that the administration of nesiritide, a recombinant form of BNP, failed to affect rehospitalization and risk of death in patients with acute decompensated heart...
failure. These findings conflicted with the generally held view that natriuretic peptides afford beneficial cardiac and hemodynamic effects. We questioned whether the lack of clinical efficacy of natriuretic peptides may derive from enhanced sympathetic activation and NE release, given that natriuretic peptides increase intracellular cGMP, and that injection of a membrane-permeable cGMP analog increases heart rate before any changes in blood pressure.

Brain natriuretic peptide and ANP significantly enhanced the basal release of NE and DA in cardiac synaptosomes and PC12 cells with EC50's of 20 and 5 nmol/L, respectively. Notably, patients with New York Heart Association class III heart failure have mean BNP plasma levels of 2 to 3 nmol/L. Considering that BNP is produced by ventricular myocytes, it is very likely that in advanced cardiac failure high concentrations of BNP will be present at the interface between ventricular myocytes and sympathetic nerve endings and reach levels capable of promoting NE release, particularly if ischemia is present, thus exacerbating heart failure symptoms. Hence, our findings in the ex vivo heart subjected to ischemia/reperfusion, isolated sympathetic nerve endings and PC12 cells are compatible with what may occur in human pathophysiology.

Inasmuch as most actions of natriuretic peptides are mediated by the guanylyl cyclase-cGMP-PKG pathway, we investigated whether their catecholamine-releasing effects are also mediated by this signaling pathway. We found that PKG inhibition not only antagonized the catecholamine-releasing effects of BNP in cardiac synaptosomes and PC12 cells but also attenuated the BNP-induced increase in PKG activity and reduced the increase in intracellular cAMP, PKA activation, and Ca2+ content. These findings suggest that PKG activation is a pivotal step in the BNP-induced stimulation of the cAMP-PKA pathway culminating in the Ca2+-mediated exocytotic process. Indeed, PKA inhibition markedly diminished the catecholamine-releasing effect of 8-bromo-cGMP, and so did the N- and L-type Ca2+ channel inhibitors ω-CTX and nifedipine, respectively. Yet because the intracellular Ca2+ chelator BAPTA-AM also greatly reduced the catecholamine-releasing effect of 8-bromo-cGMP, the relative contribution of Ca2+ entry via voltage-gated channels and of Ca2+ release from the endoplasmic reticulum remains unclear. Activation of the phospholipase C-phosphatidylinositol-IP3 pathway could also contribute...
to the increase in intracellular Ca\textsuperscript{2+}, which promotes the NE-releasing effect of natriuretic peptides. In fact, an interaction of ANP with its C-receptor was found to activate phospholipase C via G\beta\gamma.\textsuperscript{45}

Cyclic AMP is an important intracellular messenger in the regulation of neurotransmitter release in the mesenteric vasculature.\textsuperscript{46} Among the various phosphodiesterases that hydrolyze cAMP, PDE3 is known to be inhibited by cGMP.\textsuperscript{47,48} Because we found that the proexocytotic effects of BNP are associated with a PKG-dependent increase in intracellular cAMP, PKA activity, and Ca\textsuperscript{2+} content, we questioned whether this increase might be due to a cGMP/PKG-induced inhibition of PDE3. Other investigators had reported that the guanylyl cyclase-cGMP pathway regulates cAMP levels in atrial myocytes via PDE3 inhibition.\textsuperscript{49} Indeed, we found that incubation of PC12 cells with BNP decreased PDE3 activity, and this effect was prevented by PKG inhibition. Moreover, stimulation of PDE3 markedly reduced the catecholamine-releasing effect of BNP and the associated increase in PKA activity in PC12 cells in response to BNP (100 nmol/L) in the absence (control) or presence of the guanylyl cyclase inhibitor (LY83583; 10 μmol/L) or the PKG inhibitor (Rp-8-Br-cGMPS; 0.5 μmol/L) and in response to 8-Br-cGMP (1 μmol/L). Upper strip. Representative immunoblot of PC12 cell lysate probed with anti-phosphorylated PKG antibody. Columns (means±SEM; n=4-9) represent quantitative data. * and **P<0.0012 and P=0.0004, respectively, from control by unpaired t test. ***P<0.001 from control by unpaired t test. #, ##, and ###P=0.0302, P=0.0016, and P<0.0001, respectively, from controls by unpaired t test. Protein kinase A activity in PC12 cells in response to BNP (100 nmol/L) and cGMP analogs (8-pCPT-cGMP; 3 μmol/L, 8-Br-cGMP; 1 μmol/L) in the absence or presence of PKG inhibitor (Rp-8-Br-cGMPS; 0.5 μmol/L); positive control: forskolin (10 μmol/L). Columns are means±SEM (n=4-11). ***P<0.001 from control by unpaired t test. #, ##, and ###P=0.0302, P=0.0016, and P<0.0001, respectively, from controls by unpaired t test. Protein kinase A activity in PC12 cells in response to BNP (100 nmol/L), cGMP analogs (8-pCPT-cGMP; 3 μmol/L, 8-Br-cGMP; 1 μmol/L) or forskolin (10 μmol/L) in the absence or presence of PKG inhibitor (Rp-8-Br-cGMPS; 0.5 μmol/L). Upper strip. Representative immunoblots of PC12 cell lysate probed with phosphoprotein phosphatase A antibody. Lower strips. Same immunoblots probed with anti-β-actin antibody. Columns (means±SEM; n=5-9) represent quantitative data. * and **P=0.0107 and P<0.001 from control by unpaired t test. # and ##P=0.0481 and P<0.01, respectively, from own controls by unpaired t test. NE indicates norepinephrine; DA, dopamine; BNP, brain natriuretic peptide; PKG, protein kinase G; p-VASP, vasodilator-stimulated phospho-protein phosphorylation; cGMP, cyclic GMP; cAMP, cyclic AMP; and p-PKA, protein kinase A phosphorylation.

Interestingly, PDE3 activity is significantly reduced in failing human hearts and murine hearts with chronic pressure
overload. Thus, the findings that nesiritide failed to alleviate decompensated heart failure could be explained by a further BNP-induced PDE3 inhibition. This would ultimately result in additional redundant NE release, thus preventing natriuretic peptides from correcting the symptoms of CHF.

In conclusion, our findings demonstrate that natriuretic peptides in a concentration range likely to be reached at cardiac sympathetic nerve endings in advanced CHF promote Ca\(^{2+}\)-dependent NE release via a PKG-induced inhibition of PDE3-mediated cAMP hydrolysis. Thus, despite the traditional view that natriuretic peptides are cardioprotective, our findings may help explain the conclusions of the recent large clinical trial that treatment with recombinant BNP fails to protect heart failure patients from rehospitalization and death. Indeed, long-term inhibition of PDE3 was previously shown to be associated with a 40% increase in mortality, primarily as a result of arrhythmias and sudden death, which our findings may now help ascribe to excessive sympathetic activation. The corollary of these and our investigations is that agents that preserve PDE3 function rather than inhibit it may offer an alternative approach to treat cardiac dysfunctions associated with excessive sympathetic activity.

Figure 6. The catecholamine-releasing effect of BNP depends on PDE3 inhibition. A, Phosphodiesterase type 3 activation with insulin (30 nmol/L and 100 nmol/L) in synaptosomes and PC12 cells, respectively) attenuates BNP (100 nmol/L)-induced increase in NE and DA in synaptosomes isolated from guinea pig hearts (a) and NGF-differentiated PC12 cells (b). Pretreatment with the PDE3 inhibitor cilostamide (10 \(\mu\)mol/L; Cilo) prevents the insulin-mediated attenuation of the NE-releasing effect of BNP. Columns are means (±SEM; a, \(n=12\)–16; b, \(n=10\)–17). **P < 0.001 from BNP; ### and ####P < 0.0001, respectively, from the combination of BNP, insulin, and cilostamide by unpaired t test. B, Intracellular levels of cAMP in PC12 cells in response to BNP (100 nmol/L) in the absence or presence of insulin (100 nmol/L). Pretreatment with cilostamide (10 \(\mu\)mol/L; Cilo) prevents the insulin-mediated attenuation of the cAMP-enhancing effect of BNP. Columns are means (±SEM; \(n=9\)–26). ***P < 0.0001 from control; ###P < 0.0059 from BNP; †††P = 0.0027 from the combination of BNP, insulin, and cilostamide by unpaired t test. C, Brain natriuretic peptide (100 nmol/L) decreases PDE3 activity (expressed as the rate of cAMP hydrolyzed in terms of nmol/min “mg” protein) in PC12 cells. Protein kinase G inhibitor (Rp-8-Br-cGMPS; 0.5 \(\mu\)mol/L) blocks the PDE3-inhibiting effect of BNP. Insulin (100 nmol/L) and cilostamide (10 \(\mu\)mol/L alone are controls. Combination of BNP (100 nmol/L) and insulin (100 nmol/L) blocks the PDE3-inhibiting effect of BNP. Pretreatment with cilostamide (10 \(\mu\)mol/L) restores the PDE3-inhibiting effect of BNP. Columns are means (±SEM; \(n=7\)–10). ***P < 0.0001 from control; ####P < 0.0001 from BNP; ††††P = 0.0001 from the combination of BNP and insulin by unpaired t test. D, Protein kinase A activity in PC12 cells in response to BNP (100 nmol/L) in the absence or presence of insulin (100 nmol/L). Pretreatment with cilostamide (10 \(\mu\)mol/L) prevents the insulin-mediated attenuation of the PKA-enhancing effect of BNP. Forskolin (10 \(\mu\)mol/L) is the positive control. **Upper strip**, Representative immunoblot of PC12 cell lysate probed with antiphosphorylated PKA antibody. **Lower strip**, Same immunoblot probed with anti-β-actin antibody. Columns (means ±SEM; \(n=5\)–9) represent quantitative data. ***P < 0.0001 from control; ####P < 0.0001 from BNP; ††††P < 0.0001 from the combination of BNP, insulin, and cilostamide by unpaired t test. NE indicates norepinephrine; Cilo, cilostamide; BNP, brain natriuretic peptide; DA, dopamine; cAMP, cyclic AMP; PKG, protein kinase G; and p-PKG, protein kinase G phosphorylation.
Figure 7. Natriuretic peptides elicit Ca\(^{2+}\)-dependent NE exocytosis from cardiac sympathetic nerves via a cGMP-PKG-mediated inhibition of PDE3-induced cAMP hydrolysis. NP indicates natriuretic peptides; pGC, particulate guanylyl cyclase; PKG, protein kinase G; PDE3, phosphodiesterase type 3; cAMP, cyclic AMP; PKA, protein kinase A; [Ca\(^{2+}\)]\(_i\), intracellular calcium; and NE, norepinephrine.

**Sources of Funding**

This work was supported by National Institutes of Health grants HL034215 and HL47073 and by a Pharmaceutical Research and Manufacturers of America Foundation predoctoral fellowship (to N.Y.-K. Chan). We thank Paul J. Christos, DrPH, for statistical help, partially supported by Clinical Translational Science Center grant UL1-RR024996.

**Disclosures**

None.

**References**

Natriuretic Peptides Release Catecholamines

Chan et al

Natriuretic peptides have long been viewed as compensatory hormones that are upregulated in the setting of heart failure, affording beneficial cardiac and hemodynamic effects. Yet a recent large randomized trial with recombinant B-type natriuretic peptide (natin) failed to reduce mortality or rehospitalization in heart failure patients. We tested whether unsuspected proadrenergic effects might oppose the anticipated benefits of natriuretic peptides. We report that brain natriuretic peptide increases norepinephrine release in the guinea pig heart ex vivo, an effect that is further enhanced in ischemia/reperfusion. In addition, natriuretic peptides elicit catecholamine exocytosis in sympathetic nerve terminals isolated from the guinea pig heart and in nerve growth factor–differentiated rat pheochromocytoma PC12 cells, a model of sympathetic nerve endings. This proexocytotic effect is likely due to a protein kinase G–mediated inhibition of phosphodiesterase type 3. This increases intraneuronal cyclic AMP levels and protein kinase A activity, which culminates in increased intracellular calcium and norepinephrine release. Notably, these effects occur at concentrations of natriuretic peptides reached at cardiac sympathetic nerve endings in advanced congestive heart failure. We propose that this proadrenergic action may counteract any beneficial cardiac and hemodynamic effects of increasing natriuretic peptide levels in congestive heart failure and thus explain the ineffectiveness of natin as a cardiac failure medication.
An Unsuspected Property of Natriuretic Peptides: Promotion of Calcium-Dependent Catecholamine Release Via Protein Kinase G-Mediated Phosphodiesterase Type 3 Inhibition
Noel Yan-Ki Chan, Nahid Seyedi, Kenichi Takano and Roberto Levi

_Circulation_. 2012;125:298-307; originally published online December 9, 2011;
doi: 10.1161/CIRCULATIONAHA.111.059097
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/125/2/298

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/