Natriuretic Peptide-Induced Catecholamine Release from Cardiac Sympathetic Neurons: Inhibition by Histamine H3 and H4 Receptor Activation

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Received July 25, 2012; accepted August 23, 2012

ABSTRACT

We reported previously that natriuretic peptides, including brain natriuretic peptide (BNP), promote norepinephrine release from cardiac sympathetic nerves and dopamine release from differentiated pheochromocytoma PC12 cells. These proexocytotic effects are mediated by an increase in intracellular calcium secondary to cAMP/protein kinase A (PKA) activation caused by a protein kinase G (PKG)-mediated inhibition of phosphodiesterase type 3 (PDE3). The purpose of the present study was to search for novel means to prevent the proadrenergic effects of natriuretic peptides. For this, we focused our attention on neuronal inhibitory Gα/i-coupled histamine H3 and H4 receptors. Our findings show that activation of neuronal H3 and H4 receptors inhibits the release of catecholamines elicited by BNP in cardiac synaptosomes and differentiated PC12 cells. This effect results from a decrease in intracellular Ca2+ due to reduced intracellular cAMP/PKA activity, caused by H3 and H4 receptor-mediated PKG inhibition and consequent PDE3-inhibited increase in cAMP metabolism. Indeed, selective H3 and H4 receptor agonists each synergized with a PKG inhibitor and a PDE3 activator in attenuating BNP-induced norepinephrine release from cardiac sympathetic nerve endings. This indicates that PKG inhibition and PDE3 stimulation are pivotal for the H3 and H4 receptor-mediated attenuation of BNP-induced catecholamine release. Cardiac sympathetic overstimulation is characteristic of advanced heart failure, which was recently found not to be improved by the administration of recombinant BNP (nesiritide), despite the predicted beneficial effects of natriuretic peptides. Because excessive catecholamine release is likely to offset the desirable effects of natriuretic peptides, our findings suggest novel means to alleviate their adverse effects and improve their therapeutic potential.

Introduction

Although natriuretic peptides have been viewed as a compensatory neurohormonal system that is up-regulated in the setting of heart failure, affording beneficial cardiac and hemodynamic effects via particulate guanylyl cyclase stimulation and increased cGMP formation (Molkentin, 2003; Munagala et al., 2004), their role in alleviating cardiac ailments has been challenged (Wang et al., 2004; Simon et al., 2008). Indeed, in a recent large clinical trial, the administration of nesiritide [recombinant brain natriuretic peptide (BNP)] was found not to protect patients with acute heart failure (O’Connor et al., 2011).

We had reported previously that BNP promotes norepinephrine (NE) release in the guinea pig heart ex vivo, an effect that is further enhanced in ischemia/reperfusion (Chan et al., 2012). We also found that natriuretic peptides, sodium nitroprusside, and cell-permeable cGMP analogs all elicit catecholamine exocytosis in sympathetic nerves isolated from the guinea pig heart (i.e., cardiac synaptosomes) and in nerve growth factor (NGF)-differentiated PC12 cells. This proexocytotic effect 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 (Chan et al., 2012).

ABBRVIATIONS: BNP, brain natriuretic peptide; ANOVA, analysis of variance; DA, dopamine; 8-Br-cGMP, 8-bromoguanosine-3',5'-cyclic monophosphate; H3R, H3 receptor; H4R, H4 receptor; HBS, HEPES-buffered saline solution; NE, norepinephrine; NGF, nerve growth factor; PDE3, phosphodiesterase type 3; PKA, protein kinase A; PKG, protein kinase G; Rp-8-Br-cGMPS, 8-bromoguanosine-3',5'-cyclic monophosphorothioate; VASP, vasodilator-stimulated phosphoprotein; JNJ5207852, 1-{3-[4-(piperidin-1-ylmethyl)phenoxyl]propyl}piperidine; A943931, 4-((3R)-3-amino-pyrrolidin-1-yl)-6,7-dihydro-5H-benzo[6,7]cyclohepta[1,2-d]pyrimidin-2-ylamine.
More recently, it was reported that BNP increases heart rate in mice by activating the guanylyl cyclase-linked natriuretic peptide A and B receptors and inhibiting PDE3 activity, resulting in an increase in L-type Ca\textsuperscript{2+} current (Springer et al., 2012). An association of BNP with cardiac sympathetic overdrive, originating from altered Ca\textsuperscript{2+} handling and culminating in ventricular arrhythmia, was also recently described in mice (Thirieu et al., 2012).

Thus, it is conceivable that the proadrenergic effects of natriuretic peptides may offset their beneficial hemodynamic effects, as implied by the findings that β-adrenoceptor blockade protects the heart from the deleterious effects of BNP (Fujimura et al., 2009; Thireau et al., 2012). Given that an ade protects the heart from the deleterious effects of BNP effects, as implied by the findings that natriuretic peptides may offset their beneficial hemodynamic consequences (Scho¨mig, 1990; Meredith et al., 1991; Levi and Smith, 2000; Grassi et al., 2009), we investigated novel means to reduce the NE-releasing effect of natriuretic peptides, hoping that they might eventually enable a safe and effective treatment of congestive heart failure with natriuretic peptides. For this, we focused our attention on neuronal histamine H\textsubscript{3} receptors, which are G\textsubscript{q/11}-coupled and effectively inhibit physiologic and arrhythmogenic consequences (Schöming, 1990; Meredith et al., 1991; Levi and Smith, 2000). Likewise, histamine H\textsubscript{4} receptors are also G\textsubscript{q/11}-coupled (Nijmeijer et al., 2012) and seem to be present in central and peripheral neurons (Nakaya et al., 2004; Connolly et al., 2009). Therefore, we ascertained the presence of H\textsubscript{4} receptors in cardiac sympathetic nerve terminals and investigated their possible modulation of BNP-induced NE release.

We report that the activation of neuronal H\textsubscript{3} and H\textsubscript{4} receptors inhibits the release of catecholamines elicited by BNP, and this effect results from a decrease in intracellular Ca\textsuperscript{2+}. This process involves a decrease in intracellular cAMP and PKA activity, based on H\textsubscript{3} and H\textsubscript{4} receptor-mediated PKG inhibition and consequent PDE3-induced increase in cAMP metabolism.

**Materials and Methods**

**NE Release from Cardiac Synaptosomes.** Male Hartley guinea pigs weighing 300 to 350 g (Charles River Laboratories, Inc., Wilmington, MA) were killed by cervical dissection under light anesthesia with CO\textsubscript{2} vapor in accordance with institutional guidelines. The ribcage was dissected away, and the heart was rapidly perfused with Ringer’s solution at 37°C saturated with 5% CO\textsubscript{2} and 95% O\textsubscript{2}. The ribcage was dissected away, and the heart was rapidly perfused with a Langendorff apparatus. Spontaneously beating hearts were perfused through the aorta for 15 min at constant pressure (40 cm of H\textsubscript{2}O) with Ringer’s solution at 37°C saturated with 5% CO\textsubscript{2} and 95% O\textsubscript{2}. Ringer’s solution composition was 154 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl\textsubscript{2}, 6.0 mM NaHCO\textsubscript{3}, and 5.6 mM dextrose. This procedure ensured that no blood traces remained in the coronary circulation. At the end of the perfusion, the hearts were minced in ice-cold HEPES-buffered saline solution (HBS), which contained 50 mM HEPES, pH 7.4, 144 mM NaCl, 5 mM KCl, 1.2 mM CaCl\textsubscript{2}, 1.2 mM MgCl\textsubscript{2}, and 10 mM glucose. Synaptosomes were isolated as described previously (Seyedi et al., 1997). Minced tissue was digested with 40 mg of collagenase (type II; Worthington Biochemicals, Freehold, NJ) per 10 ml of HBS per gram of wet heart weight for 1 h at 37°C. HBS contained 1 mM pargyline to prevent enzymatic destruction of NE. After low-speed centrifugation (10 min at 120g and 4°C), the resulting pellet was suspended in 10 volumes of 0.32 M sucrose and homogenized with a Teflon/glass homogenizer. The homogenate was spun at 650g for 10 min at 4°C, and the pellet was then rehomogenized and respun. The pellet containing cellular debris was discarded, and the supernatants from the last two spins were combined and equally subdivided into tubes. Each tube was centrifuged for 20 min at 20,000g at 4°C. This pellet, which contained cardiac synaptosomes, was resuspended in HBS to a final volume of 1 ml in a water bath at 37°C. Each suspension functioned as an independent sample and was used only once. In every experiment, one sample was untreated (control, basal NE release), and others were incubated with BNP for 10 min. When drugs were used, synaptosomes were preincubated with drugs for 10 min. When antagonists were used, samples were preincubated with the antagonists before incubation with the agonist. Controls were incubated for an equivalent length of time without drugs. At the end of the incubation period, each sample was centrifuged (20 min, 20,000g, 4°C). The supernatant was assayed for NE content by high-pressure liquid chromatography with electrochemical detection (Seyedi et al., 1997). The pellet was assayed for protein content by a modified Lowry procedure (Seyedi et al., 1997).

**Cell Culture.** Rat pheochromocytoma PC12 cells were transfected with the human histamine H\textsubscript{3} receptor (donated by Dr. T. W. Lovenberg, Johnson and Johnson Pharmaceutical R&D, LLC, San Diego, CA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. PC12-H\textsubscript{3} cell lines were selected and maintained in medium containing 500 μg/ml G-418 sulfate (Medi-tech, Herndon, VA). PC12 and PC12-H\textsubscript{3} cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum, 5% donor horse serum, 1% l-glutamine, and antibiotics at 37°C in 5% CO\textsubscript{2}. The differentiating protocol involved plating PC12 and PC12-H\textsubscript{3} cells on tissue culture plates coated with collagen (rat tail type VII, Sigma-Aldrich, St. Louis, MO) combined with exposure to low serum medium containing 1% fetal bovine serum, 0.5% donor horse serum, 1% l-glutamine, and antibiotics supplemented with 78-NGF (BD Biosciences Discovery Labware, Bedford, MA). For each experiment, the culture medium was aspirated and cells were washed twice with Na-Ringer’s (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM MgCl\textsubscript{2}, 2 mM glucose, and 2 mM CaCl\textsubscript{2}), then incubated with BNP (100 nM), for 20 min in an incubator at 37°C either in the absence or presence of methimipex (histamine H\textsubscript{3} receptor agonist; 1 μM) (Kitbunnadaj et al., 2005), 4-methylhistamine (histamine H\textsubscript{4} receptor agonist; 20 μM) (Lim et al., 2005), 1-[3-[4-(piperidin-1-ylmethyl)phenoxy]propyl]piperidine (JNJ5207852) (histamine H\textsubscript{4} receptor antagonist; 30 nM) (Barbie et al., 2004), or 4-((3R)-3-amino- pyrrolidin-1-yl)-6,7-dihydro-5H-benzo[6,7]cyclohepta[1,2-d]pyrimidin-2-ylamine (A943931) (histamine H\textsubscript{3} receptor antagonist; 300 nM) (Cowart et al., 2008). When these drugs were used, PC12-H\textsubscript{3} cells were preincubated with them for 10 min. 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-min treatment with Triton X-100) were taken from each well and analyzed for dopamine (DA) content by high-performance liquid chromatography with electrochemical detection with a 6-min retention time. Other cell lysates were analyzed for histamine H\textsubscript{3} and H\textsubscript{4} receptor expression by Western blotting, intracellular cAMP levels, PKA activity, intracellular Ca\textsuperscript{2+}, PKG activity, or PDE3 activity.

**Intracellular Ca\textsuperscript{2+} Assay.** Cells were washed twice with Na-Ringer’s, and then treated with potassium (100 mM; 3 min) or BNP (100 nM; 10 min) in the presence or absence of methimipex (histamine H\textsubscript{3} receptor agonist; 1 nM), 4-methylhistamine (histamine H\textsubscript{4} receptor agonist; 20 μM), JNJ5207852 (histamine H\textsubscript{4} receptor antagonist; 30 nM), or A943931 (histamine H\textsubscript{3} receptor antagonist; 300 nM). Controls were incubated for an equivalent length of time without drugs. At the end of each experiment, cells were washed with Dulbecco’s phosphate-buffered saline containing 10 mM EGTA to chelate external Ca\textsuperscript{2+} and then with normal phosphate-buffered saline to remove the remaining EGTA. Cells were then lysed with the addition of water and harvested with a scraper. Ca\textsuperscript{2+} content was determined by using a Ca\textsuperscript{2+} assay kit (QuantiChrom Ca\textsuperscript{2+} Assay Kit, BioAssay Systems, Hayward, CA). The Ca\textsuperscript{2+} content was adjusted by
the protein content of the cells and expressed as milligrams of Ca$^{2+}$ per milligram of protein.

### cAMP Assay
Cells were treated and lysed as described above. Intracellular cAMP levels were determined by using a cAMP Biotrak EIA kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) following the manufacturer’s protocol. This cAMP assay is highly specific and 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 below 0.01%, whereas cAMP is 100%.

### PKA Activity
PKA phosphorylation (an indication of PKA activation) was measured by using a phosphorylated-PKA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in Western blot. Methods for Western blot analysis were as described previously (Chan et al., 2012).

### 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 (Gill et al., 2007). VASP phosphorylation (i.e., PKG activity) was measured by using a phosphorylated-VASP antibody (Santa Cruz Biotechnology, Inc.) in Western blot. Methods for Western blot analysis were as described previously (Chan et al., 2012).

### PDE3 Activity
PDE3 activity was measured by using a commercially available colorimetric PDE assay kit (Enzo Life Sciences, Farmingdale, NY) as described previously (Chan et al., 2012). Cell lysates were prepared and then total protein concentration was measured as described above. Free phosphate contamination was removed according to the manufacturer’s protocol. Samples were incubated for 10 min at 37°C, and reactions were stopped with Biomol Green (Enzo). Samples were then put on a shaker for 20 min at room temperature. Results were measured by using a microplate reader (Molecular Devices, Sunnyvale, CA). PDE3-specific cAMP-hydrolytic activity was expressed as the difference between cAMP hydrolyzed (expressed as nmol/min/mg protein) in the presence and absence of the specific PDE3 inhibitor cilostamide.

### Drugs and Chemicals
BNP was purchased from AnaSpec, Inc. (Fremont, CA); 8-Br-cGMP, forskolin, Rp-8-Br-cGMPs, insulin, and cilostamide were purchased from Sigma-Aldrich. Methimepip, JNJ5207852, 4-methyl histamine, and A943931 were purchased from Tocris Bioscience (Ellisville, MO).

#### Statistics
Data are presented as mean ± S.E.M. Parametric tests were used throughout the study. Either unpaired t test or one-way ANOVA followed by post hoc Dunnett’s test was used in all figures. GraphPad Prism version 4.03 for Windows (GraphPad Software, Inc., San Diego, CA) was used. Values of P < 0.05 were considered statistically significant.

### Results

#### K$^+$- and BNP-Induced Norepinephrine Release from Cardiac Sympathetic Nerve Endings: Attenuation by Histamine H$_3$ and H$_4$ Receptor Activation.

Depolarization of isolated cardiac synaptosomes with extracellular potassium (100 mM) elicited a ~25% increase in NE release (Fig. 1, A and B). In the presence of the histamine H$_3$ receptor agonist methimepip (1 nM) (Kitbunnadaj et al., 2005) the K$^+$-induced increase in NE release was reduced by ~50%, an effect that was abolished by the selective H$_3$ receptor antagonist JNJ5207852 (30 nM) (Barbier et al., 2004) (Fig. 1A). The K$^+$-induced increase in NE release was also attenuated by ~54% by the selective H$_4$ receptor agonist 4-methylhistamine (20 μM) (Lim et al., 2005) (Fig. 1B). This effect was abolished by the selective H$_4$ receptor antagonist A943931 (300 nM) (Cowart et al., 2008) (Fig. 1B).

Incubation of isolated cardiac synaptosomes with BNP (100 nM; 10 min) elicited a ~25 to 28% increase in NE release (Fig. 1, C and D). In the presence of the selective H$_4$ receptor agonist methimepip (1 nM) (Fig. 1C) or the selective H$_4$ receptor agonist 4-methylhistamine (20 μM), respectively. Each agonist was used either alone or together with the respective selective antagonist, JNJ5207852 (H$_3$ receptor antagonist; 30 nM) and A943931 (H$_4$ receptor antagonist; 300 nM). Bars represent mean increases in NE release above basal level (± S.E.M.; n = 8 and 12 for A and B, respectively). Basal NE level was 255.4 ± 16.8 pmol/mg of protein (n = 36). *, P < 0.01 from corresponding control by one-way ANOVA followed by post hoc Dunnett’s test. C and D, release of endogenous NE from guinea pig heart synaptosomes by human BNP (100 nM; 10-min exposure) in the absence and presence of the selective H$_3$ receptor agonist methimepip (1 nM) or the selective H$_4$ receptor agonist 4-methylhistamine (20 μM), respectively. Each agonist was used either alone or together with the respective selective antagonist as in A and B. Bars represent mean increases in NE release above basal level (± S.E.M.; n = 12 for C and D, respectively). Basal NE level was 279.6 ± 9.3 pmol/mg of protein (n = 36). **, P < 0.01 from corresponding control by one-way ANOVA followed by post hoc Dunnett’s test.

![Fig. 1](image-url)
induced depolarization or the administration of a natriuretic peptide.

**BNP-Induced Dopamine Release from PC12 and PC12-H₃ Cells: Attenuation by Histamine H₃ and H₄ Receptor Activation.** To investigate possible mechanisms of the H₃ and H₄ receptor-mediated attenuation of the NE-releasing effect of natriuretic peptides, we used the rat pheochromocytoma PC12 cell line. These cells, once differentiated with NGF, exhibit a sympathetic nerve-ending phenotype (Chan et al., 2012) and constitutively express only the H₄ receptor (Fig. 2). We also used a PC12 cell line stably transfected with the H₃ receptor (PC12-H₃) (Morrey et al., 2008) (Fig. 2). Dopamine is the endogenous catecholamine in both cell types (Morrey et al., 2008).

Incubation of PC12 and PC12-H₃ cells with BNP (100 nM, 20 min) elicited a ~48% increase in endogenous DA release (Fig. 3). In the presence of the selective H₃ receptor agonist methimepip (1 nM) (Fig. 3A) or the selective H₄ receptor agonist 4-methylhistamine (20 μM) (Fig. 3B), the BNP-induced increase in DA release was inhibited by ~90% in each case (Fig. 3). This inhibition was abolished by the selective H₃ receptor antagonist JNJ5207852 in PC12-H₃ cells (Fig. 3A) and the selective H₄ receptor antagonist A943931 in PC12 cells (Fig. 3B). In contrast, the H₃ receptor agonist methimepip, either alone or in the presence of the H₄ receptor antagonist JNJ5207852, failed to modify the BNP-induced increase in DA release in PC12 cells, which do not express H₃ receptors (i.e., negative control; Fig. 3B).

![Fig. 2. Expression of histamine H₃ and H₄ receptors in PC12 and PC12-H₃ cells.](image)

**BNP Increases cAMP and Activates PKA in PC12 and PC12-H₃ Cells: Attenuation by Histamine H₃ and H₄ Receptor Activation.** Incubation of PC12-H₃ cells with BNP (100 nM) caused a ~2-fold increase in the intracellular concentration of cAMP (compared with a ~10-fold increase by 10 μM forskolin, positive control). The BNP-induced increase in cAMP was inhibited by ~50% by the selective H₄ receptor agonist methimepip (1 nM), a result that was abolished by the selective H₃ receptor antagonist JNJ5207852 (30 nM) (Fig. 4A). BNP also activated PKA, as evidenced by a ~60% increase in PKA phosphorylation (similar to that elicited by forskolin used as positive control; Fig. 4B). The BNP-induced increase in PKA activity was also inhibited by ~50% by the selective H₄ receptor agonist methimepip (1 nM), a result that was abolished by the selective H₃ receptor antagonist JNJ5207852 (30 nM) (Fig. 4B).

Incubation of PC12 cells with BNP (100 nM) caused a ~3-fold increase in the intracellular concentration of cAMP (compared with a ~15-fold increase by 10 μM forskolin, positive control). The BNP-induced increase in cAMP was inhibited by ~60% by the selective H₄ receptor agonist 4-methylhistamine (20 μM), a result that was abolished by the selective H₃ receptor antagonist A943931 (300 nM) (Fig. 4C). In contrast, H₃ receptor activation with methimepip (1 nM) did not affect the BNP-induced increase in cAMP in these cells, which did not express H₃ receptors (negative control) (Fig. 4C). The BNP-induced increase in PKA activity was also inhibited by ~50% by the selective H₄ receptor agonist 4-methylhistamine (20 μM), an effect that was abolished by the selective H₄ receptor antagonist A943931 (300 nM) (Fig. 4D). H₃ receptor activation with methimepip did not affect the BNP-induced increase in PKA activity (negative control) (Fig. 4D).

**BNP Increases Intracellular Ca²⁺ in PC12 and PC12-H₃ Cells: Attenuation by Histamine H₃ and H₄ Receptor Activation.** Depolarization of PC12 and PC12-H₃ cells with K⁺ (100 mM) increased intracellular Ca²⁺ concentration ~2.5- and 5-fold, respectively (positive control). Incubation with BNP (100 nM) also increased intracellular Ca²⁺ concentration 2- and 4-fold, respectively (Fig. 5). The effect of BNP was reduced by ~40 and ~65% in the presence of the H₃ receptor agonist 4-methylhistamine (20 μM) and the H₄ receptor agonist methimepip (1 nM) in PC12 and PC12-H₃ cells, respectively (Fig. 5). Methimepip (negative control) did not affect the BNP-induced increase in intracellular Ca²⁺ (Fig. 5).

![Fig. 3. BNP-induced dopamine release in PC12 cells: inhibition by histamine H₃ and H₄ receptor activation.](image)
not affect the BNP-induced increase in intracellular Ca²⁺ in PC12 cells (Fig. 5B). The H₃ and H₄ receptor-mediated inhibition of the BNP-induced increase in intracellular Ca²⁺ was abolished by the respective H₃ and H₄ receptor antagonists [JNJ5207852 (30 nM) and A943931 (300 nM)] (Fig. 5).

**BNP-Induced Increase in PKG Activity in PC12 and PC12-H₃ Cells: Attenuation by Histamine H₃ and H₄ Receptor Activation.** Incubation of PC12-H₃ cells with either 8-Br-cGMPS (1 μM; positive control) or BNP (100 nM) elicited a ~50% increase in PKG activity, which was prevented either by the PKG inhibitor Rp-8-Br-cGMPS (0.5 μM) or the H₄ receptor agonist 4-methylhistamine (20 μM); 4-methylhistamine’s effect was abolished by the H₄ receptor antagonist A943931 (300 nM) (Fig. 6B). Methimepip did not affect the BNP-induced increase in PKG activity in PC12 cells, which do not constitutively express H₃ receptors (negative control) (Fig. 6B).

To further assess the role of diminished PKG activity in the H₃ and H₄ receptor-mediated attenuation of BNP-induced catecholamine exocytosis, we next determined whether a synergistic effect could be seen when H₃ and H₄ receptor activation was combined with PKG inhibition. As shown in Fig. 6C, when either methimepip or Rp-8-Br-cGMPS was used at subthreshold concentrations (0.03 nM and 0.3 μM, respectively), neither...
caused a significant diminution of BNP-induced (100 nM) NE release in cardiac synaptosomes. In contrast, a significant attenuation occurred when the same subthreshold concentrations of methimepip and Rp-8-Br-cGMPS were combined (Fig. 6C). Likewise, when either 4-methylhistamine or Rp-8-Br-cGMPS was used at subthreshold concentrations (0.03 and 10 nM, respectively), neither caused a significant diminution of BNP-induced (100 nM) NE release in cardiac synaptosomes. In contrast, a significant attenuation occurred when the same subthreshold concentrations of methimepip and insulin were combined (Fig. 7C). Likewise, when either 4-methylhistamine or insulin was used at subthreshold concentrations (0.03 μM and 10 nM, respectively), neither caused a significant diminution of BNP-induced (100 nM) NE release in cardiac synaptosomes. In contrast, a significant attenuation occurred when the same subthreshold concentrations of 4-methylhistamine and insulin were combined (Fig. 7D).

These synergistic responses suggested that an increase in PDE3 activity is likely to be involved in the H₃ and H₄ receptor-mediated attenuation of BNP-induced catecholamine exocytosis.

**Discussion**

The purpose of our study was to search for novel means to prevent the recently uncovered proadrenergic effects of natriuretic peptides. Our findings indicate that the activation of neuronal histamine H₃ and H₄ receptors attenuates BNP-induced catecholamine release by inhibiting PKG, thus enhancing PDE3-mediated cAMP metabolism culminating in a decrease in intracellular Ca²⁺.

Although H₄ receptors are expressed predominantly in hematopoietic cells (Nijmeijer et al., 2012), their presence had been reported in the brain (Zhu et al., 2001; Connelly et al., 2009) and peripheral neurons of the nasal mucosa (Nakaya et al., 2004). Here, we functionally identified H₄ receptors in cardiac sympathetic neurons and demonstrated their protein expression in NGF-differentiated PC12 cells exhibiting a sympathetic neuron phenotype. It is noteworthy that differentiated PC12 cells constitutively expressed only H₄ receptors. We further demonstrated that, similar to H₃ receptors, these neuronal H₄ receptors negatively modulate catecholamine exocytosis elicited by K⁺-induced depolarization or BNP. Given that H₄ receptors are highly homologous to H₃ receptors and that, like H₃ receptors, are coupled to inhibitory Gᵢₒ proteins (Oda et al., 2000; Liu et al., 2001; Zhu et al., 2001), it was not surprising to find that H₄ receptors attenuate catecholamine exocytosis elicited by K⁺ depolarization. As is the case for H₃ receptors, the antieocytotic action of H₄ receptors could result from a Go₃-mediated impairment of the adenyl cyclase-cAMP-PKA pathway lead-
Fig. 6. A and B, histamine H_{3} and H_{4} receptor activation inhibits the increase in PKG activity elicited by BNP in PC12-H_{3} and PC12 cells, respectively. C and D, H_{3} and H_{4} receptor activation synergizes with PKG inhibition in attenuating BNP-induced NE release in cardiac synaptosomes, respectively. A, PKG activity in PC12-H_{3} cells treated with 8-Br-cGMP (1 μM; positive control) or BNP (100 nM) in the absence or presence of the PKG inhibitor Rp-8-Br-cGMPS (0.5 μM) or the H_{3} receptor agonist methimepip (1 nM), either alone or in combination with the H_{3} receptor antagonist JNJ5207852 (30 nM). Top band, representative immunoblot of PC12-H_{3} cell lysate probed with antiphosphorylated VASP (p-VASP; a major PKG substrate) antibody. Bottom band, same immunoblot probed with anti-β-actin antibody. Bars represent mean quantitative values (± S.E.M.; n = 4). Significantly different from control: ***, P < 0.001 by unpaired t test. Significantly different from BNP: †††, P < 0.001 by unpaired t test. Significantly different from BNP + H_{3} receptor agonist + H_{4} receptor antagonist: ###, P < 0.001 by unpaired t test. B, PKG activity in PC12 cells treated with 8-Br-cGMP (1 μM; positive control) or BNP (100 nM) in the absence or presence of the PKG inhibitor Rp-8-Br-cGMPS (0.5 μM) or the H_{4} receptor agonist (4-methylhistamine; 20 μM), either alone or in combination with the H_{4} receptor antagonist (A943931; 300 nM). The H_{4} receptor agonist methimepip (1 nM; negative control) failed to modify the response to BNP in PC12 cells, which do not constitutionally express H_{4} receptors. Top band, representative immunoblot of PC12 cell lysate probed with antiphosphorylated VASP antibody. Bottom band, same immunoblot probed with anti-β-actin antibody. Bars represent mean quantitative values (± S.E.M.; n = 4). Significantly different from control: ***, P < 0.001 and **, P < 0.01 by unpaired t test. Significantly different from BNP: ††, P < 0.01 by unpaired t test. Significantly different from BNP + H_{4}R agonist + H_{4}R antagonist: ###, P < 0.01 by unpaired t test. C, inhibition of BNP (100 nM)-induced NE release in cardiac synaptosomes by subthreshold concentrations of the PKG inhibitor Rp-8-Br-cGMPS (0.3 μM) and the H_{4} receptor agonist methimepip (0.03 nM), administered either alone or in combination. D, inhibition of BNP-induced NE release in cardiac synaptosomes by subthreshold concentrations of the PKG inhibitor Rp-8-Br-cGMPS and the H_{4} receptor agonist 4-methyl histamine (0.03 μM), administered either alone or in combination. Note in C and D that a significant attenuation of NE release occurs when the PKG inhibitor is combined either with the H_{3} or the H_{4} receptor agonist (⁎, P < 0.05 and ††, P < 0.005 by unpaired t test). Bars, means ± S.E.M. (n = 8–18), represent the BNP-induced increase in NE release above the basal level of 221.1 ± 8.9 pmol/mg (n = 23).

We found that H_{3} and H_{4} receptor activation synergized with PKG inhibition and PDE3 stimulation, respectively, in inhibiting the BNP-induced promotion of catecholamine release. These synergistic responses strongly suggest that a decrease in PKG activity and an increase in PDE3 activity are both pivotal in the H_{3} and H_{4} receptor-mediated attenuation of BNP-induced catecholamine exocytosis. Whether PDE3 activation indirectly results from PKG inhibition because of H_{3} and H_{4} receptor activation or is a direct and independent target of H_{3} and H_{4} receptor activation remains to be understood.

We can only speculate at this point on the molecular mechanisms possibly involved in H_{3} and H_{4} receptor-mediated PKG inhibition and PDE3 stimulation. We had shown previously that imetit, a mixed H_{3}/H_{4} receptor agonist (Morse et al., 2001; Zhu et al., 2001), attenuates the phorbol ester-induced activation of PKC in NGF-differentiated PC12-H_{3} cells, an action prevented by the mixed H_{3}/H_{4} receptor antagonist clobenpropit (Hashikawa-Hobara et al., 2012). Be-
PC12-H3 cells

A

PC12 cells

B

Cardiac synaptosomes

C

D

Fig. 7. A and B, histamine H₃ and H₄ receptor activation inhibits the decrease in PDE3 activity (expressed as rate of cAMP hydrolyzed) elicited by BNP in PC12-H3 and PC12 cells. C and D, H₃ and H₄ receptor activation synergizes with PDE3 activation in attenuating BNP-induced NE release in cardiac synaptosomes. A, BNP (100 nM) decreases the rate of cAMP hydrolyzed (i.e., a decrease in PDE3 activity) in PC12-H3 cells. The H₃ receptor agonist methimepip (1 nM) reverses the PDE3-inhibiting effect of BNP. Pretreatment with the H₃ receptor antagonist JNJ5207852 (30 nM) restores the PDE3-inhibiting effect of BNP. The PDE3 activator insulin (100 nM) and the PDE3 inhibitor cilostamide (10 μM) serve as controls. C, inhibition of NE release induced by BNP (100 nM) in cardiac synaptosomes by subthreshold concentrations of the PDE3 activator insulin (10 nM) and the H₃ receptor agonist methimepip (0.03 nM). D, inhibition of NE release induced by BNP (100 nM) in cardiac synaptosomes by subthreshold concentrations of the PDE3 activator insulin (10 nM) and the H₄ receptor agonist 4-methylhistamine (0.03 μM) administered either alone or in combination. Note in C and D that a significant attenuation of NE release occurs when insulin is combined either with the H₃ or the H₄ receptor agonist (**, P < 0.005 by unpaired t test). Bars, means ± S.E.M. (n = 8–19), represent BNP-induced increase in NE release above the basal level of 226.9 ± 12.9 pmol/mg (n = 21).

cause PKC stimulation has been reported previously to activate PKG (Hou et al., 2003), it is conceivable that the H₃/H₄ receptor-induced decrease in PKC activity could in turn reduce PKG activity in cardiac sympathetic neurons. Reduced PKG activity would then alleviate PKG-mediated PDE3 inhibition, augment cAMP hydrolysis, and ultimately decrease intracellular Ca²⁺ and NE exocytosis. It is also possible that the activation of H₃ and H₄ receptors may lead to PDE3 stimulation independently of PKG inhibition. In fact, H₃ receptors are known to activate the phosphatidylinositol 3-kinase pathway, which results in the phosphorylation/activation of Akt (Leurs et al., 2005). Akt is involved in the phosphorylation/activation of PDE3B (Wijkander et al., 1998), which was shown to be expressed in heart tissue with PDE3A (Liu and Maurice, 1998). Given the high homology of H₃ receptors to H₄ receptors, and the fact that they both are Gₛα-coupled (Nijmeijer et al., 2012), it is conceivable that H₃ and H₄ receptor activation may lead via the phosphatidylinositol 3-kinase pathway to PDE3 stimulation, increased cAMP hydrolysis, and decreased NE release.

It is noteworthy that PDE3 activity is significantly reduced in failing human hearts and murine hearts with chronic pressure overload (Ding et al., 2005). Moreover, long-term inhibition of PDE3 has been found to be associated with a 40% increase in mortality, primarily as a result of arrhythmias and sudden death (Packer et al., 1991; Nony et al., 1994). We had reported that inhibition of PDE3-mediated cAMP hydrolysis by natriuretic peptides, at concentrations likely to be reached in advanced congestive heart failure, promote excessive NE release (Chan et al., 2012), which we contend could explain at least in part why natriuretic peptides failed to correct the symptoms of congestive heart failure (O’Connor et al., 2011). Thus, we had
advocated that agents that preserve PDE3 function, rather than inhibiting it, may be beneficial in the treatment of cardiac dysfunctions associated with excessive sympathetic activity (Chan et al., 2012). We report here that histamine H3 and H4 receptor activation stimulates PDE3 activity via PKG inhibition and/or directly. Accordingly, preserving and/or stimulating PDE3 function via H3 and H4 receptor activation could offer a useful new approach to the treatment of cardiac dysfunctions with natriuretic peptides. Indeed, although β-adrenoceptor blockade has been advocated to prevent the deleterious effects of chronic BNP exposure in congestive heart failure (Thireau et al., 2012), stimulation of PDE3 activity via H3 and H4 receptor activation might be preferable, given the notorious adverse effects of β-blockers (Lewis and McDevitt, 1986).

In conclusion, we had reported previously that natriuretic peptides augment NE exocytosis from cardiac sympathetic neurons by a PKG-mediated inhibition of PDE3 activity, which results sequentially in an increase in intraneuronal cAMP, augmented PKA activity, phosphorylation of Ca2+ channels, and increased intracellular Ca2+. (Chan et al., 2012). We present new evidence that this pathway can be effectively interrupted at the PKG and PDE3 levels. Indeed, our findings indicate that PKG and PDE3 are targeted for inhibition and stimulation, respectively, when histamine H3 and H4 receptors are activated (see Fig. 8).

Cardiac sympathetic overstimulation is characteristic of advanced heart failure (Esler and Kaye, 2000; Braunwald, 2008; Grassi et al., 2009), which was recently found not to be improved by the administration of recombinant BNP (nesiritide) (O’Connor et al., 2011), despite the predicated beneficial effects of natriuretic peptides (Molkentin, 2003; Munagala et al., 2004). Because excessive NE release is likely to offset the desirable effects of natriuretic peptides, our findings suggest novel means to alleviate their adverse effects and improve their therapeutic potential.

Acknowledgments

The data presented in Fig. 1 were obtained in experiments performed by Dr. N. Seyerdi. We thank Dr. Kenichi Takano for help with figure digitization.

Authorship Contributions

Participated in research design: Chan, Robador, and Levi.
Conducted experiments: Chan and Robador.
Performed data analysis: Chan, Robador, and Levi.
Wrote or contributed to the writing of the manuscript: Chan, Robador, and Levi.

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Fig. 8. Histamine H3 and H4 receptor activation inhibits Ca2+-dependent NE exocytosis from cardiac sympathetic nerves via inhibition of PKG and consequently reduction of PKG-dependent PDE3 inhibition. NP, natriuretic peptides; pGC, particulate guanylyl cyclase; cGMP, cyclic GMP; [Ca2+]i, intracellular calcium.


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