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

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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 G/H₉ 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-induced 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, which bear a sympathetic nerve-ending phenotype (Chan et al., 2012). 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).

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ABBREVIATIONS: 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)phenoxy]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^{2+} current (Springer et al., 2012). An association of BNP with cardiac sympathetic overdrive, originating from altered Ca^{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; Thirieu et al., 2012). Given that an enhanced NE release bears dysfunctional and arrhythmogenic consequences (Schömig, 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. For this, we focused our attention on neuronal histamine H₃ receptors, which are Go_{i/o}-coupled and effectively inhibit physiologic and pathophysiologic NE release (Imamura et al., 1995; Seyed et al., 1997; Levi and Smith, 2000). Likewise, histamine H₄ receptors are also Go_{i/o}-coupled (Nijmeijer et al., 2012) and seem to be present in central and peripheral neurons (Nakaya et al., 2004; Connelly et al., 2009). Therefore, we ascertained the presence of H₃ receptors in cardiac sympathetic nerve terminals and investigated their possible modulation of BNP-induced NE release.

We report that the activation of neuronal H₃ and H₄ receptors inhibits the release of catecholamines elicited by BNP, and this effect results from a decrease in intracellular Ca^{2+}. This process involves a decrease in intracellular cAMP and PKA activity, based on H₃ and H₄ receptor-mediated PKG inhibition and consequent PDE3-increased 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 dislocation under light anesthesia with CO₂ vapor in accordance with institutional guidelines. The ribcage was dissected away, and the heart was rapidly excised, freed from fat and connective tissue, and transferred to a Langendorff apparatus. Spontaneously beating hearts were perfused through the aorta for 15 min at constant pressure (40 cm of H₂O) with Ringer’s solution at 37°C saturated with 5% CO₂ and 95% O₂, 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 CaCl₂, 1.2 mM MgCl₂, and 10 mM glucose. Synaptosomes were isolated as described previously (Seyed 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 incubated 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 H₃ 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-H₃ cell lines were selected and maintained in selection media containing 500 μg/ml G-418 sulfate (Mediatech, Herndon, VA). PC12 and PC12-H₃ 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% CO₂. The differentiating protocol involved plating PC12 and PC12-H₃ 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 MgCl₂, 2 mM glucose, and 2 mM CaCl₂), then incubated with BNP (100 nM), for 20 min in an incubator at 37°C either in the absence or presence of methimipex (histamine H₃ receptor agonist; 1 μM) (Kitbunnadaj et al., 2005), 4-methylhistamine (histamine H₄ receptor agonist; 20 μM) (Lim et al., 2005), 1-[3-(4-(piperidin-1-ylmethyl)phenoxy)propyl]piperidine (JNJ5207852) (histamine H₄ receptor antagonist; 30 nM) (Barlier et al., 2004), or 4-((3R)-3-amino-pyrrolidin-1-yl)-6,7-dihydroxy-5H-benzo[6,7]cyclohepta[1,2-d]pyrimidin-2-ylamine (A943931) (histamine H₄ receptor antagonist; 300 nM) (Cowart et al., 2008). When these drugs were used, PC12-H₃ cells were preincubated with them for 10 min. Controls were incubated for an equivalent length of time with 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 H₃ and H₄ receptor expression by Western blotting, intracellular Ca^{2+}, PKA activity, or PDE3 activity.

Intracellular Ca^{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 H₃ receptor agonist; 1 nM), 4-methylhistamine (histamine H₄ receptor agonist; 20 μM), JNJ5207852 (histamine H₄ receptor antagonist; 30 nM), or A943931 (histamine H₄ 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^{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^{2+} content was determined by using a Ca^{2+} assay kit (Quantichrom Chrom Ca^{2+} Assay Kit; BioAssay Systems, Hayward, CA). The Ca^{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-cGMP, 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_3 receptor agonist 4-methylhistamine (20 μM) (Lim et al., 2005) (Fig. 1B). This effect was abolished by the selective H_3 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_3 receptor agonist methimepip (1 nM) (Fig. 1C) or the selective H_4 receptor agonist 4-methylhistamine (20 μM) (Fig. 1D) the BNP-induced increase in NE release was reduced by ~58 and 52%, respectively (Fig. 1, C and D). These effects were abolished by the selective H_3 receptor antagonist JNJ5207852 (30 nM) (Fig. 1C) and the selective H_4 receptor antagonist A943931 (300 nM) (Fig. 1D), respectively.

These findings indicated the presence not only of histamine H_3 receptors in cardiac sympathetic nerve endings (Seyedi et al., 2005) but also H_4 receptors, both of which are capable of attenuating the release of NE elicited by K^+ -
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 H₄ receptor agonist methimepip, either alone or in the presence of the H₃ receptor agonist 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).

![Image](74x318 to 266x416)

**Fig. 2.** Expression of histamine H₃ and H₄ receptors in PC12 and PC12-H₃ cells. Top, Western blotting analysis of HEL 92.1.7 cells (Liu et al., 2001; Zhu et al., 2001) (HEL 92.1.7 are human erythroleukemia cells, in which H₃ receptors are highly expressed, and thus serve as positive control) and PC12 cells, both expressing histamine H₃ receptors. Bottom, Western blotting analysis of PC12 and PC12-H₃ cells showing expression of histamine H₃ receptors in PC12-H₃ cells but not in PC12 cells. Twenty micrograms of total proteins were loaded in each lane.

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**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 H₃ 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 H₃ receptor agonist methimepip (1 nM), a result that was abolished by the selective H₄ 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 H₄ receptor antagonist A943931 (300 nM) (Fig. 4C). In contrast, H₄ 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 PKA activity (negative control) (Fig. 4D).

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**Fig. 3.** BNP-induced dopamine release in PC12 cells: inhibition by histamine H₃ and H₄ receptor activation. A, dopamine release elicited by BNP (100 nM) in PC12-H₃ cells, in the absence or presence of the selective H₃ receptor agonist methimepip (1 nM), either alone or in combination with the selective H₄ receptor agonist JNJ5207852 (30 nM). Columns are means ± S.E.M. (n = 7–10). Significantly different from BNP and BNP + H₄ receptor agonist: ##, P < 0.01 and ###, P < 0.001, respectively, by unpaired t test. B, dopamine release elicited by BNP (100 nM) in PC12 cells, in the absence or presence of the selective H₄ receptor agonist 4-methyl histamine (20 μM), either alone or in combination with the selective H₃ receptor antagonist A943931 (300 nM). Columns are means ± S.E.M. (n = 8). †††, significantly different from BNP and BNP + H₃ receptor agonist: H₃R receptor antagonist (P < 0.0001 by unpaired t test).

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**Fig. 4.** BNP-induced increase in intracellular cAMP and PKA activity in PC12 and PC12-H₃ cells. A, intracellular cAMP levels in PC12-H₃ cells, in the absence or presence of the selective H₃ receptor agonist methimepip (1 nM) or the selective H₄ receptor agonist 4-methylhistamine (20 μM), either alone or in combination with the selective H₄ receptor agonist JNJ5207852 (30 nM). Columns are means ± S.E.M. (n = 7–10). Significantly different from BNP and BNP + H₄ receptor agonist: ##, P < 0.01 and ###, P < 0.001, respectively, by unpaired t test. B, PKA activity in PC12 cells, in the absence or presence of the selective H₄ receptor agonist 4-methylhistamine (20 μM) and the selective H₃ receptor agonist methimepip (1 nM), either alone or in combination with the selective H₃ receptor antagonist A943931 (300 nM). Columns are means ± S.E.M. (n = 8). †††, significantly different from BNP and BNP + H₃ receptor agonist: H₃R receptor antagonist (P < 0.0001 by unpaired t test).
Histamine H₃ receptor activation attenuates the increase in intracellular cAMP and phosphorylation of PKA (PKA activity) elicited by BNP in PC12-H₃ cells. Histamine H₃ receptor activation attenuates the increase in intracellular cAMP and phosphorylation of PKA elicited by BNP in PC12 cells. A, intracellular cAMP levels in PC12-H₃ cells treated with forskolin (10 μM; positive control) or BNP (100 nM) in the absence or presence of the selective H₃ receptor agonist methimepip (1 nM), either alone or in combination with the selective H₃ receptor antagonist JNJ5207852 (30 nM). Columns are means ± S.E.M. (n = 4). Significantly different from control: **, P < 0.01 by unpaired t test. Significantly different from BNP: ##, P < 0.01 by unpaired t test. B, PKA activity in PC12-H₃ cells treated with forskolin (10 μM; positive control) or BNP (100 nM) in the absence or presence of the H₃ receptor antagonist methimepip (1 nM), either alone or in combination with the H₃ receptor antagonist JNJ5207852 (30 nM). Top band, representative immunoblot of PC12 cell lysate probed with antiphosphorylated PKA 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 < 0.01 by unpaired t test. Significantly different from BNP and BNP + H₄ receptor agonist + H₃ receptor antagonist: †, P < 0.01 by unpaired t test. C, intracellular cAMP levels in PC12 cells treated with forskolin (10 μM; positive control) or BNP (100 nM) in the absence or presence of the H₃ receptor agonist methimepip (1 nM); negative control) or H₄ receptor agonist 4-methylhistamine (20 μM), either alone or in combination with the H₃ receptor antagonist A943931 (300 nM). Columns are means ± S.E.M. (n = 4). Significantly different from control: **, P < 0.01 and ###, P < 0.001 by unpaired t test. Significantly different from BNP: ##, P < 0.01 by unpaired t test. Significantly different from BNP + H₃ receptor agonist + H₄ receptor antagonist: †, P < 0.05 by unpaired t test. D, PKA activity in PC12 cells treated with forskolin (10 μM; positive control) or BNP (100 nM) in the absence or presence of the H₃ receptor agonist methimepip (1 nM) or H₄ receptor agonist 4-methylhistamine (20 μM), either alone or in combination with the H₃ receptor antagonist A943931 (300 nM). Top band, representative immunoblot of PC12 cell lysate probed with antiphosphorylated PKA 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 < 0.01 and ###, P < 0.001 by unpaired t test. Significantly different from BNP: ##, P < 0.01 by unpaired t test.

not affect the BNP-induced increase in intracellular Ca²⁺ in PC12 cells (Fig. 5B). The H₄ and H₄ receptor-mediated inhibition of the BNP-induced increase in intracellular Ca²⁺ was abolished by the respective H₄ 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-cGMP (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 H₃ 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 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 4-methylhistamine 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 H₃ 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 Gi/o 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 antieexocytotic action of H₄ receptors could result from a Goi-mediated impairment of the adenyl cyclase-cAMP-PKA pathway lead-
different from BNP

8-Br-cGMP (1 μM). Top band, representative immunoblot of PC12-H3 cell lysate probed with antiphosphorylated VASP (p-VASP; a major PKG

anti-VASP 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 < 0.01 by unpaired t test. Significantly different from BNP + H3 receptor agonist + H4 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 H3 receptor agonist methimepip (0.03 nM), either alone or in combination with the H4 receptor antagonist (A943931; 300 nM). The H3 receptor agonist methimepip (1 nM; negative control) failed to modify the response to BNP in PC12 cells, which do not constitutionally express H3 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 < 0.05 by unpaired t test. Significantly different from BNP: ††, P < 0.01 by unpaired t test. Significantly different from BNP + H4R agonist + H4R 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 H4 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 H3 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 H3 or the H4 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 22.1 ± 8.9 pmol/mg (n = 25).

Fig. 6. A and B, histamine H3- and H4 receptor activation inhibits the increase in PKG activity elicited by BNP in PC12-H3 and PC12 cells, respectively. C and D, H3 and H4 receptor activation synergizes with PKG inhibition in attenuating BNP-induced NE release in cardiac synaptosomes, respectively. A, PKG activity in PC12-H3 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 H3 receptor agonist methimepip (1 nM), either alone or in combination with the H4 receptor antagonist (JNJ507852; 30 nM). Top band, representative immunoblot of PC12-H3 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 < 0.01 by unpaired t test. Significantly different from BNP + H4R agonist: ##, 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 H4 receptor agonist (4-methyl histamine; 20 μM), either alone or in combination with the H3 receptor antagonist (A943931; 300 nM). The H3 receptor agonist methimepip (1 nM; negative control) failed to modify the response to BNP in PC12 cells, which do not constitutionally express H3 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 < 0.05 by unpaired t test. Significantly different from BNP: ††, P < 0.01 by unpaired t test. Significantly different from BNP + H3R agonist + H4R 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 H4 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 H3 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 H3 or the H4 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 22.1 ± 8.9 pmol/mg (n = 25).

ing to a decrease in intraneuronal Ca2+ (Silver et al., 2002; Seyedi et al., 2005). A direct Gβγ-induced attenuation of Ca2+ current (IcA) could also play a role (Morrey et al., 2008). On the other hand, regarding the H3 and H4 receptor-mediated attenuation of catecholamine release elicited by BNP, our findings suggest that the reduction in intracellular Ca2+ derives primarily from PKG inhibition and a consequent enhancement in PDE3-mediated cAMP metabolism, rather than a Gαq-mediated decrease in adenyl cyclase activity.

We found that H3 and H4 receptor activation synergized with PKG inhibition and PDE3 stimulation, respectively, in inhibiting the BNP-induced promotion of catecholamine release. These synergic responses strongly suggest that a decrease in PKG activity and an increase in PDE3 activity are both pivotal in the H3 and H4 receptor-mediated attenuation of BNP-induced catecholamine exocytosis. Whether PDE3 activation indirectly results from PKG inhibition because of H3 and H4 receptor activation or is a direct and independent target of H3 and H4 receptor activation remains to be understood.

We can only speculate at this point on the molecular mechanisms possibly involved in H3 and H4 receptor-mediated PKG inhibition and PDE3 stimulation. We had shown previously that imetit, a mixed H3/H4 receptor agonist (Morse et al., 2001; Zhu et al., 2001), attenuates the phorbol ester-induced activation of PKC in NGF-differentiated PC12-H3 cells, an action prevented by the mixed H3/H4 receptor antagonist clobenpropit (Hashikawa-Hobara et al., 2012). Be-
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 PKC activity, 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 PKC activity, 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, 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 Gαo-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.

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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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