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Efficacy of B-Type Natriuretic Peptide Is Coupled to Phosphodiesterase 2A in Cardiac Sympathetic Neurons

Dan Li, **Chieh-Ju Lu**, **Guoliang Hao**, **Hannah Wright**, **Lavinia Woodward**, **Kun Liu**, **Elisa Vergari**, **Nicoletta C. Surdo**, **Neil Herring**, **Manuela Zaccolo**, and **David J. Paterson**
Burdon Sanderson Cardiac Science Centre, Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom.

Abstract

Elevated B-type natriuretic peptide (BNP) regulates cGMP-phosphodiesterase activity. Its elevation is regarded as an early compensatory response to cardiac failure where it can facilitate sympathovagal balance and cardiorenal homeostasis. However, recent reports suggest a paradoxical proadrenergic action of BNP. Because phosphodiesterase activity is altered in cardiovascular disease, we tested the hypothesis that BNP might lose its efficacy by minimizing the action of cGMP on downstream pathways coupled to neurotransmission. BNP decreased norepinephrine release from atrial preparations in response to field stimulation and also significantly reduced the heart rate responses to sympathetic nerve stimulation in vitro. Using electrophysiological recording and fluorescence imaging, BNP also reduced the depolarization evoked calcium current and intracellular calcium transient in isolated cardiac sympathetic neurons. Pharmacological manipulations suggested that the reduction in the calcium transient was regulated by a cGMP/protein kinase G pathway. Fluorescence resonance energy transfer measurements for cAMP, and an immunoassay for cGMP, showed that BNP increased cGMP, but not cAMP. In addition, overexpression of phosphodiesterase 2A after adenoviral gene transfer markedly decreased BNP stimulation of cGMP and abrogated the BNP responses to the calcium current, intracellular calcium transient, and neurotransmitter release. These effects were reversed on inhibition of phosphodiesterase 2A. Moreover, phosphodiesterase 2A activity was significantly elevated in stellate neurons from the prohypertensive rat compared with the normotensive control. Our data suggest that abnormally high levels of phosphodiesterase 2A may provide a brake against the inhibitory action of BNP on sympathetic transmission.

Keywords

calcium; natriuretic peptide, brain; sympathetic nervous system; synaptic transmission

Correspondence to Dan Li or David J. Paterson, Department of Physiology, Anatomy and Genetics, University of Oxford, Sherrington Bldg, Parks Rd, Oxford OX1 3PT, United Kingdom. dan.li@dpag.ox.ac.uk or david.paterson@dpag.ox.ac.uk.

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None.

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Natriuretic peptides (NPs) are generally regarded as cardioprotective, owing to their antihypertrophic properties,[1] ability to reduce plasma volume,[2,3] and beneficial action on neurohumoral control.[4] B-type or brain natriuretic peptide (BNP) has recently received much attention because of its diagnostic, prognostic, and potentially therapeutic role in heart failure,[5] acute ischemic stroke,[6] and hypertension,[7] although its therapeutic efficacy is still not firmly established.[8]

The physiological effects of NPs are elicited through binding to the particulate guanylyl cyclase–coupled natriuretic peptide receptors (NPRs).[9] This results in increases in $cGMP^{3}$-dependent modulation of protein kinase G (PKG)[10] and cyclic nucleotide–coupled phosphodiesterases[11] that leads to the regulation of intracellular calcium concentrations ($[Ca^{2+}]_i$).[12] Upregulation of NP production is seen as a compensatory mechanism affording beneficial hemodynamic[13] and myocardial effects,[14,15] reflected in their ability to slow the progression of heart failure[16] and also inhibit cardiac sympathetic responsiveness in these patients.[17] The importance of targeting the cardiac sympathetic nervous system is now seen as a major therapeutic opportunity.[18] However, the paracrine action of NP on the sympathetic nervous system is controversial.[19]

Indeed Chan et al[20] suggested that a paradoxical proadrenergic action of BNP, via PKG-mediated inhibition of cAMP hydrolysis by PDE3 and subsequent Ca^{2+}-dependent norepinephrine release, may account for the lack of clinical efficacy of NPs. This seemed surprising given the volume of literature that indicates an increase in cGMP caused by nitric oxide–activated soluble guanylyl cyclase reduces $[Ca^{2+}]_i$ and cardiac sympathetic neurotransmission.[21–23]

Because PDE2A activity is dysregulated in heart failure,[24] we tested the hypothesis that BNP loses its efficacy by impairing the action of cGMP on a calcium-regulated pathway coupled to exocytosis. Here, we demonstrate that BNP suppresses the neuronal calcium current and $[Ca^{2+}]_i$ transients in primary cultured sympathetic neurons from stellate ganglia that predominately innervate the heart.[25] It also reduces cardiac [^{3}H]-norepinephrine release and heart rate responses to intact cardiac sympathetic nerve stimulation. Moreover, PDE2A activity is upregulated in stellate neurons from prehypertensive spontaneously hypertensive rats (SHR) versus normotensive Wistar-Kyoto rats. Overexpressing PDE2A decreases cGMP and the efficacy of BNP to regulate Ca^{2+}-induced exocytosis.

Materials and Methods

Animals

Four-week-old male Sprague Dawley (n=142), prehypertensive SHR (n=8), and normotensive Wistar-Kyoto (n=8) rats were used in this study. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Animals (Scientific Procedures) Act 1986 (United Kingdom).

An expanded Materials and Methods section is available in the online-only Data Supplement.

Results

BNP Decreases Heart Rate Responses to Right Stellate Stimulation In Vitro and Evoked Norepinephrine Release

We asked whether BNP could directly regulate the heart rate responses to cardiac sympathetic nerve stimulation in an isolated innervated double atria preparation that was decentralized and devoid of circulating factors. Stimulation of the right stellate ganglion produced a frequency-dependent increase in heart rate (Figure 1Ai). Addition of 250 nmol/L BNP caused no significant changes in baseline heart rate (control, 309±16 bpm; BNP, 311±14 bpm; P=0.91, paired t test), whereas it caused a significantly reduced heart rate response to nerve stimulation across all frequencies when compared with control stimulations (n=10; Figure 1Ai and 1Aii). There was a trend for a lower concentration (100 nmol/L) at 1 and 3 Hz to also decrease sympathetic responsiveness (Figure S1 in the online-only Data Supplement).

To test whether the attenuated heart rate response to nerve stimulation caused by BNP was a result of decreased neurotransmission, we directly measured the level of [^{3}H]-norepinephrine release in response to field stimulation of right atrial preparations. Because only 250 nmol/L reached a statistically reduced heart rate response to nerve stimulation, we chose to use 250 nmol/L on the [^{3}H]-norepinephrine release experiment. There was no difference between the first (S1) and second (S2) field stimulation at 5 Hz without BNP (S1, +1.68±0.12%; S2, +1.64±0.15%; n=4; Figure 1Bi and 1Biii), indicating no significant time-dependent changes. However, as we expected, BNP (250 nmol/L) produced ≈43% reduction in [^{3}H]-norepinephrine release in response to 5-Hz field stimulation (S1, +1.75±0.25%; S2, +0.99±0.23%; n=6; P<0.05, 1-way ANOVA; Figure 1Bii and 1Biii).

BNP Reduces Neuronal Calcium Current and Intracellular-Free Calcium Transients in Cardiac Sympathetic Neurons

We next assessed whether BNP would also affect calcium signaling in isolated stellate neurons. Antityrosine hydroxylase immunofluorescence staining from fixed cultured stellate neuron is shown in Figure 2A. Virtually all the neurons had tyrosine hydroxylase–positive expression, confirming that the neurons were sympathetic. The neuronal calcium current was measured using the whole cell configuration of the patch-clamp technique. The calcium current was significantly reduced by −16.7±5.9% (P<0.05; n=6) and −15.2±3.2% (P<0.05; n=3) after 10-minute exposure to 100 and 250 nmol/L BNP, respectively (Figure 2Bi and 2Bii; Figure S2), and the amplitude was recovered after washing off (at −10 mV, the maximum response). Intracellular calcium concentration was measured using ratiometric recordings (with Fura-2-acetoxymethyl ester, Fura-2/AM) in single sympathetic neurons. A protocol for the calcium-transient response to 50 mmol/L of KCl is shown in Figure 2Ci. BNP was introduced at 7 minutes after the first high K^+ stimulation (S1; Figure 2Ci). After 10 minutes of treatment, neurons were stimulated again (S2) in the presence of BNP. In a concentration-dependent manner, BNP (1, 10, 50, 100, 250 nmol/L) caused ≈2% to 22% decrease in $[Ca^{2+}]_i$ evoked by high K^+ depolarization from cardiac sympathetic neurons. This was statistically significant at 100 and 250 nmol/L (Figure 2Cii), however, these concentrations did not affect basal $[Ca^{2+}]_i$ (Figure 2Ciii). There was no significant

difference between 100 and 250 nmol/L BNP on $[Ca^{2+}]_i$, so we chose to use 100 nmol/L in the cellular experiments.

Pharmacological Manipulation of NPR-A/cGMP/PKG Pathway

Several putatively selective inhibitors of the NPR-A/cGMP/PKG pathway were used to further elucidate the signal transduction pathway (Figure 3). Pretreatment of NPR-A inhibitor Isatin (1H-indole-2,3-dione, 10 μmol/L) or the PKG inhibitor (RP-8-Br-PET-cGMP, 5 μmol/L) almost completely abolished the action of 100 nmol/L BNP on the $[Ca^{2+}]_i$. cAMP-dependent protein kinase inhibitor (1 μmol/L) alone reduced the $[Ca^{2+}]_i$ transient (−12.5±3.2% compared with control). However, BNP maintained its ability to reduce the depolarization evoked $[Ca^{2+}]_i$ transient in the presence of the protein kinase inhibitor, suggesting protein kinase was not critical to the pathway downstream after NPR-A activation.

Phosphodiesterases play a major role in cell signaling by hydrolyzing cAMP and cGMP.[26] A nonspecific phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), was tested. IBMX of 100 and 500 μmol/L reduced the $[Ca^{2+}]_i$ transient by −8.8±3.4% and −16.7±2.8%, respectively. This indicated that the overall phosphodiesterases are more effective at hydrolyzing cGMP in the sympathetic neurons. Moreover, 100 nmol/L BNP further diminished the $[Ca^{2+}]_i$ transient in the presence of IBMX at both concentrations (Figure 3C). In the nervous system, the isoforms PDE2 and PDE3 are dominant.[20,27] Therefore, the PDE3 inhibitor Milrinone (10 μmol/L) or PDE2 inhibitor Bay60-7550 (1 μmol/L) was introduced during BNP stimulation. In the presence of Milrinone, BNP still maintained its inhibition on the $[Ca^{2+}]_i$ transient (Figure 3D). Interestingly, application of BNP in the presence of Bay60-7550 further reduced the $[Ca^{2+}]_i$ transient compared with BNP alone (Figure 3D).

BNP Does Not Affect Intracellular cAMP Level

Real-time measurements of intracellular cAMP concentration in living cardiac sympathetic neurons were first performed using ratiometric fluorescence resonance energy transfer (FRET) imaging in the presence of BNP. Depolarizing the neuron with 50 mmol/L KCl led to a decrease in yellow fluorescent protein emission (Figure 4A, yellow trace) and a concomitant increase in cyan fluorescent protein emission (Figure 4A, cyan trace), resulting in an increase of the ratiometric FRET signal ($\Delta R/R_0$). This is consistent with an increase in cAMP accumulation.[28] Neurons were stimulated again in the presence of 100 nmol/L BNP (Figure 4A and 4B). BNP caused no significant changes in the FRET signal (Figure 4C). Moreover, in the unstimulated neuron, 100 and 250 nmol/L BNP did not cause a significant decrease in the FRET signal (P=0.35 and 0.19, respectively, n=4; Figure 4D). PDE2 inhibitor Bay60-7550 (1 μmol/L) also did not significantly affect the unstimulated FRET signal (Figure 4E, n=4). To show that the sensitivity of the FRET sensor was intact, application of IBMX (100 μmol/L) caused a marked rise in cAMP (Figure 4E).

Effect of PDE2A Overexpression in Presence of BNP

Increased Stellate Ganglia PDE2A Activity From Prehypertensive SHR and PDE2A Overexpression—Because the depolarization induced $[Ca^{2+}]_i$ transient in

stellate neurons is enhanced in prehypertensive SHRs[29] and reduced after application with PDE2A inhibition in normal cells, we measured the PDE2A activity in these stellate ganglia. As shown in Figure 5A, PDE2 activity (expressed as the cGMP-PDE2 hydrolytic activity) was ≈60% higher in the SHR when compared with the Wistar-Kyoto rats (Figure 5Ai). Moreover, it was ≈90% greater in PDE2A transduced ganglia (Figure 5Aii) compared with the empty treated group, as we attempted to mimic the SHR PDE levels.

Western Blotting and cGMP Production—Gene transfer of empty or Ad.PDE2A to the isolated stellate ganglia was confirmed with Western blot analysis. PDE2A. mCherry expression (127 KDa, with anti-PDE2A antibody) was significantly enhanced in Ad.PDE2A transduced stellate ganglia tissue when compared with those transduced with the empty virus alone (Figure 5B). We detected no difference in endogenous levels of PDE2A (100 KDa) protein in either group (n=5 in each group; Figure S3). cGMP production (Figure 5C) was markedly enhanced (≈3-fold) after application of 250 nmol/L BNP in the transduced empty virus stellate ganglia tissue. However, in the presence of BNP, overexpression of Ad.PDE2A reduced cGMP accumulation by ≈60% compared with the empty control transduced tissue. This suggests that PDE2A is responsible for the degradation of newly synthesized BNP-activated cGMP.

PDE2A Overexpression Increases Neuronal Calcium Current and Intracellular-Free Calcium Transients—Fluorescence microscopy detected mCherry expression in stellate neurons after transduction with the empty virus or Ad.PDE2A (Figure 6B). Because all neurons did not express mCherry, we only selected the transduced neurons to measure calcium current and intracellular calcium transients. The calcium current was significantly reduced by ≈21% after 10-minute exposure to 100 nmol/L BNP (Figure 6Ai) in the empty transduced group. Whereas in Ad.PDE2A transduced neurons, BNP failed to affect the calcium current (Figure 6Aii). This response was recovered after inhibition of PDE2 by Bay60-7550 (1 μmol/L) in Ad.PDE2A transduced neurons (reduced ≈12%, Figure 6Aiii).

As we expected, $[Ca^{2+}]_i$ transients were significantly enhanced (≈32%) in Ad.PDE2A transduced neurons when compared with the empty control (Figure 6Ci and 6Cii). BNP of 100 nmol/L reduced the $[Ca^{2+}]_i$ transient by −18.6±3.8% in the empty group which was comparable with results from cultured stellate neurons without virus (−18.1±3.6%). By contrast, BNP in Ad.PDE2A transduced neurons failed to affect the $[Ca^{2+}]_i$ transient (−4.99±3.09%) compared with empty. This response was rescued after inhibition of PDE2 by Bay60-7550 (1 μmol/L), where BNP then reduced the $[Ca^{2+}]_i$ tran sient by −23.1±6.4% (Figure 6Ciii).

Evoked Norepinephrine Release—We then investigated whether overexpression of PDE2A affected neurotransmitter release. [^{3}H]-Norepinephrine release during 5-Hz field stimulation of right atrial preparations was significantly enhanced (S1, ≈37%) in Ad.PDE2A transduced tissue when compared with the empty control (Figure 7A). BNP produced ≈28% reduction in [^{3}H]-norepinephrine release in the empty vector control group (Figure 7Ai and 7Aiii). However, BNP did not change [^{3}H]-norepinephrine release after transduction with Ad.PDE2A (S2 versus S1, Figure 7Aii and 7Aiii). Percutaneous gene transfer to the right atrium was confirmed with Western blot analysis (Figure 7B) by

detecting enhanced PDE2A.mCherry expression (127 KDa, with antimCherry antibody) compared with those transduced with the empty virus.

Discussion

In this study, we provide some insight into the inhibitory action of BNP signaling on cardiac sympathetic transmission and excitability. We found that BNP reduces $[Ca^{2+}]_i$ transients in a concentration-dependent manner and also decreases the calcium current in primary cultured stellate cells. This translated into a reduction in neurotransmitter release and heart rate responsiveness to direct sympathetic nerve stimulation in vitro. The inhibitory action of BNP on the $[Ca^{2+}]_i$ transient is regulated by the NPR-A/cGMP/PKG pathway, where PDE2 modulates the hydrolysis of cGMP that is synthesized by the addition of BNP (Figure 8). Where PDE2A activity is elevated in stellate neurons, it is conceivable that it provides a brake against the inhibitory action of BNP on sympathetic transmission.

BNP Suppresses Cardiac Sympathetic Nerve Activity

Our findings agree with the generally held view that NPs afford beneficial cardiac and hemodynamic effects.[16] NPs have been viewed as a compensatory neurohormonal system that is upregulated in the setting of heart failure[30] and hypertension.[31] Specifically, infusion of BNP decreases norepinephrine spillover in patients with heart failure,[17] and in experimental animal models, atrial NP produces a direct depressant action on sympathetic nerve function by reducing both cardiac output and arterial blood pressure.[32] Atrial NP also augments cardiac parasympathetic nerve activity in humans[33] and rats,[34] and of interest, NP mimic the action of the nitric oxide–cGMP pathway in facilitating acetylcholine release.[35] Moreover, elevating cGMP levels with nitric oxide inhibits calcium transients and sympathetic neurotransmission, indicating a pivotal role for this cyclic nucleotide in the modulation of neurotransmission.[21,22,29] In this work, we found that BNP decreased norepinephrine release from atrial preparations in response to field stimulation and also significantly reduced the heart rate responses to sympathetic nerve stimulation in vitro. Taken together, these studies would suggest a potentially positive synergistic effect on sympathovagal signaling that might be beneficial in the setting of chronically increased sympathetic and decreased parasympathetic drive.

BNP Inhibition of $[Ca^{2+}]_i$ Transient Are Regulated by NPR-A/cGMP/PKG Pathway

Although BNP reduces the calcium current and $[Ca^{2+}]_i$ transients in stellate cells during depolarization, it is unlikely that the $[Ca^{2+}]_i$ transient is only affected by membrane calcium entry. The endoplasmic reticulum and mitochondria also regulate intracellular calcium (ie, also sensitive to nitric oxide–cGMP levels) after cellular depolarization with KCl.[29,36] Whether BNP directly affects these subcellular organelles has not been established.

The receptor for BNP, NPR-A ,is expressed on cells in many different tissues of various organ systems.[37] NPR-A is linked to particulate guanylate cyclase, a unique intracellular guanylate cyclase-catalytic domain for this receptor that mediates the biological actions through the production of cGMP as a second messenger.[38] This was confirmed by direct measurement of cGMP levels that were increased with BNP in stellate ganglia tissue. Our

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finding that NPR-A blockade prevents BNP-mediated reductions in peak $[Ca^{2+}]_i$ gives functional evidence for NPR-A binding mediating the effects of BNP. Failure of BNP to reduce Ca^{2+} transients when the PKG blocker was added confirmed that BNP evoked reductions in $[Ca^{2+}]_i$ transients were most likely dependent on PKG-mediated inhibition of voltage-gated Ca^{2+} channels. This is consistent with the proposal that BNP activates a PKG signaling pathway involved in the inhibition of neurotransmission in the dorsal root ganglion.[39]

PKG is known to be activated by cGMP, the activity of which is also regulated by phosphodiesterases.[26] Our results differ from a previous study that highlights a pivotal role for protein kinase in mediating the paradoxical proadrenergic effects of BNP. Here, it was shown that PKG-mediated inhibition of cAMP hydrolysis by PDE3 caused a subsequent Ca^{2+}-dependent release of norepinephrine.[20] This conclusion was based on experiments performed on a PC12 cell line in the absence of neuronal membrane depolarization. In contrast, our results in depolarized cardiac sympathetic neurons showed that inhibition of PDE3 and protein kinase did not disrupt the effect of BNP on the $[Ca^{2+}]_i$ transient. Moreover, BNP had virtually no effect on the FRET measured cAMP under either basal conditions or during periods of stimulation. In addition, the inhibitory effect of BNP on the $[Ca^{2+}]_i$ transient was augmented by blocking PDE2, but prevented by inhibition of PKG. This result is consistent with others who report that in primary neuronal cultures (from the brain), inhibition of PDE2 enhances cGMP levels, whereas cAMP levels are unaffected.[40]

The Role of PDE2 in Cardiac Neural Signaling

PDE2A has been shown to play important roles in many signal transduction pathways as a regulator of both cGMP and cAMP levels.[40–43] PDE2 is markedly upregulated in heart failure and blunts β-adrenergic responses by hydrolysis of cAMP in cardiomyocytes.[24,44] This provides a negative crosstalk mechanism between cAMP and cGMP signaling pathways. In cardiac stellate ganglia, we observed that the action of BNP is regulated by PDE2A activity. We also show that PDE2A activity is upregulated in the SHR stellate ganglia. This in turn depletes cGMP and reduces PKG activity, resulting in decreased inhibition of voltage-gated calcium channels and intracellular calcium transients. Our results agree with others that PDE2 predominantly hydrolyses cGMP rather than cAMP in neurons.[45,46] The difference between the myocyte and neuron may well be related to the microdomain organization of PDE2A splice variants (PDE2A1–3) expressed in difference species.[47]

The inhibitory action of BNP on the calcium current/$[Ca^{2+}]_i$ and neurotransmission was prevented by overexpression of PDE2A. This was associated with ≈60% reduction in cGMP, suggesting that PDE2A plays a key role in modulating the efficacy of BNP. Furthermore, blockade of overexpressed PDE2A re-established the action of BNP. Our data suggest a possible site of regulation on PDE2A signaling, where BNP might fail in hypertension, resulting in excessive sympathetic activity.

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Perspectives

Plasma levels of BNP increase because of chronic stretch in heart failure[3] and can rise by ≤30-fold in severe cases.[48] The finding that BNP reduced sympathetic outflow from stimulated stellate ganglion neurons is suggestive of a compensatory protective role against sympathetic overactivity, implicated in the pathophysiology of both hypertension[49] and heart failure.[50] Our findings suggest that contrary to the proposal of Chan et al,[20] the lack of clinical efficacy of BNP is not attributable to the proexocytotic nature of the BNP pathway. Instead, we propose that the usual ability of BNP to modulate Ca^{2+} signaling and neurotransmission in sympathetic neurons is via a cGMP-PDE2A pathway. BNP has a diverse action and thus an integrative view of this neurohormone at a systemic level must also be taken into account if it is to be viewed as a targeted therapy in vivo. Moreover, whether the BNP-cGMP-PDE2A pathway is actually impaired in hypertensive and heart failure models remains to be firmly established.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Significance

What Is New?

- Our results have identified a novel site of regulation on phosphodiesterase 2A signaling where BNP might fail, resulting in excessive cardiac sympathetic neurotransmission.

What Is Relevant?

- We report that phosphodiesterase 2A activity is markedly upregulated in cardiac sympathetic neurons from the prohypertensive rat and show that direct upregulation of phosphodiesterase 2A enhances sympathetic activity.

Summary

Phosphodiesterase 2A may play a key role in modulating the B-type natriuretic peptide–cGMP–phosphodiesterase pathway in the cardiac sympathetic hyper-responsiveness in hypertensive and heart failure models.

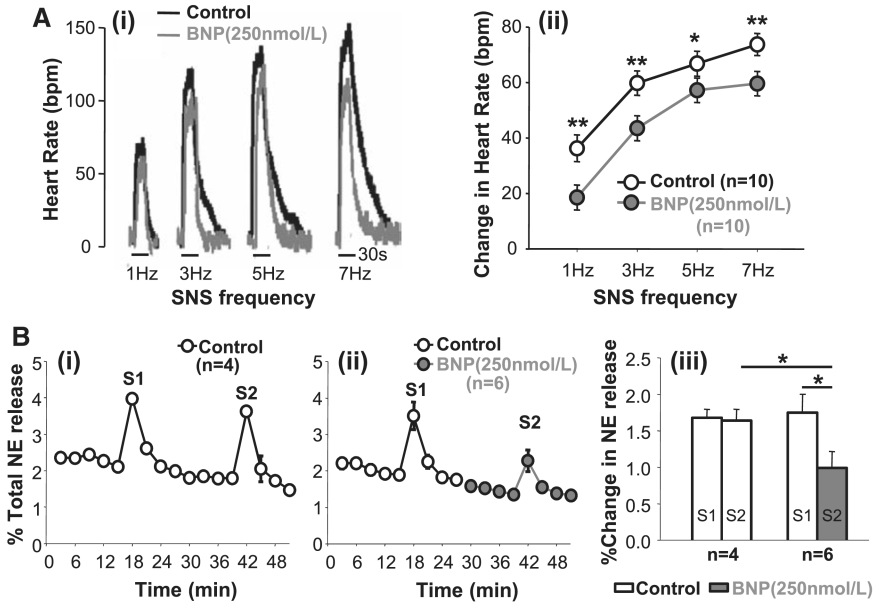

Figure 1.

A, Representative raw data traces (i) and grouped data (ii) showing the heart rate responses to sympathetic nerve stimulation (SNS) at 1, 3, 5 and 7 Hz for 30 s with 250 nmol/L B-type natriuretic peptide (BNP) compared with control. **P*<0.05, ***P*<0.01, paired *t* test. **B**, Grouped data showing the time control (i) and with addition of 250 nmol/L BNP (ii) on [^{3}H]-norepinephrine release from isolated atria. The atria were stimulated at 5 Hz for 1 minute at the 16th (S1) and 40th (S2) minutes. (iii) Group mean data show BNP caused a significant decrease in 5-Hz stimulation evoked [^{3}H]-norepinephrine release (n=6 **P*<0.05, 1-way ANOVA). NE indicates norepinephrine.

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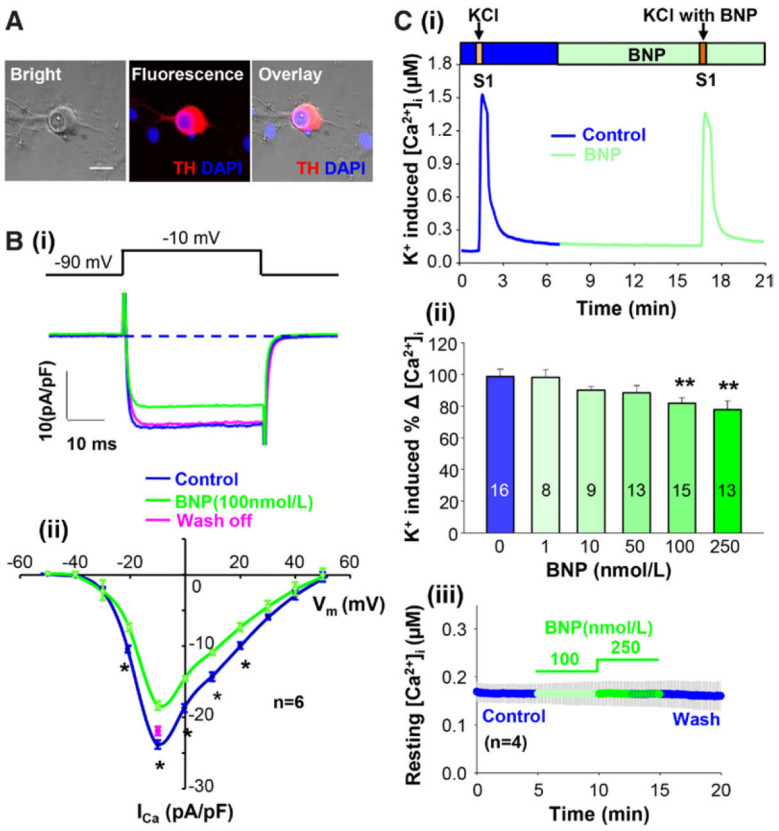

Figure 2.

A, Bright field and immunofluorescence staining image of a cultured cardiac sympathetic neuron derived from a stellate ganglion which was stained with the catecholamine neuronal marker antityrosine hydroxylase (TH, red) and costained with the nuclear marker 4′,6-diamidino-2-phenylindole (DAPI, blue). Scale bar represents 20 μm. **B**, (i) Representative whole cell calcium current density traces obtained with or without 100 nmol/L B-type natriuretic peptide (BNP, 10 minutes) and after wash out. Currents were evoked by test pulses to −10 mV from a holding potential of −90 mV. (ii) Mean current density–voltage relations in the presence and absence of 100 nmol/L BNP. Wash off data were only recorded at −10mV as the quality of the recordings deteriorates over time. **P*<0.05, paired *t* test, n=6 neurons. I_{Ca} indicates calcium current; pA, picoampere; and pF, picofarad. **C**, (i) An example recording calcium transient in a single cardiac sympathetic neuron. Neuron was exposed to KCl for 30 s to depolarize the neuron with (S2) or without (S1) BNP. (ii) Statistical data showing that concentration–effect relationship of BNP (1–250 nmol/L) changed KCl evoked increase in $[Ca^{2+}]_i$ expressed as a ratio (%) of S2 compared with S1. ***P*<0.01, compared with control, unpaired *t* test. Numbers shown in the bars indicate the number of the neurons. (iii) BNP (100 and 250 nmol/L) did not affect basal $[Ca^{2+}]_i$ in the cardiac sympathetic neurons (n=4).

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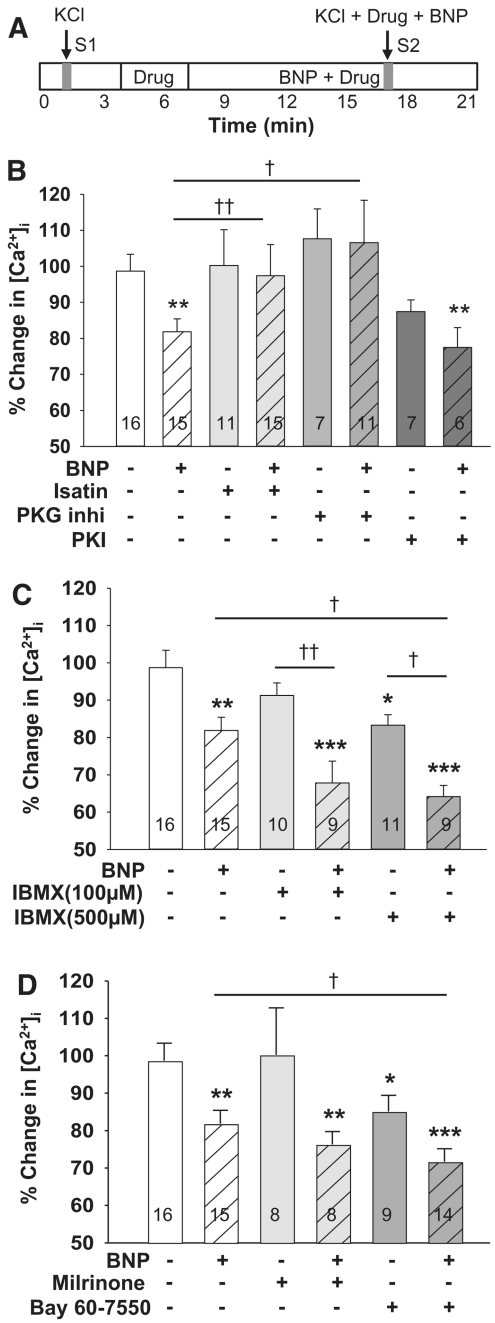

Figure 3.

NP-cGMP pathway inhibitors action on the intracellular calcium transient. **A**, Depolarization of the neuron with 50 mmol/L KCl for 30 s at 1 minute (S1, without drug) and 17 minutes (S2, with drug and 100 nmol/L BNP). **B**, Data showing the effect on the $[Ca^{2+}]_i$ of the natriuretic peptide receptor A (NPR-A) antagonist Isatin (10 μmol/L), the protein kinase G (PKG) inhibitor RP-8-Br-PET-cGMP (5 μmol/L), and the protein kinase A inhibitor (PKI; 1 μmol/L) with or without BNP; (**C**) nonspecific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 100 and 500 μmol/L) with or without BNP; and (**D**)

Phosphodiesterase 3 (PDE3) inhibitor Milrinone (10 μmol/L) or the PDE2 inhibitor Bay60-7550 (1 μmol/L) with or without BNP. **P*<0.05, ***P*<0.01, ****P*<0.001, compared with control, unpaired *t* test. †*P*<0.05, ††*P*<0.01, 1-way ANOVA. Numbers shown in the bars indicated the number of neurons. BNP indicates B-type natriuretic peptide.

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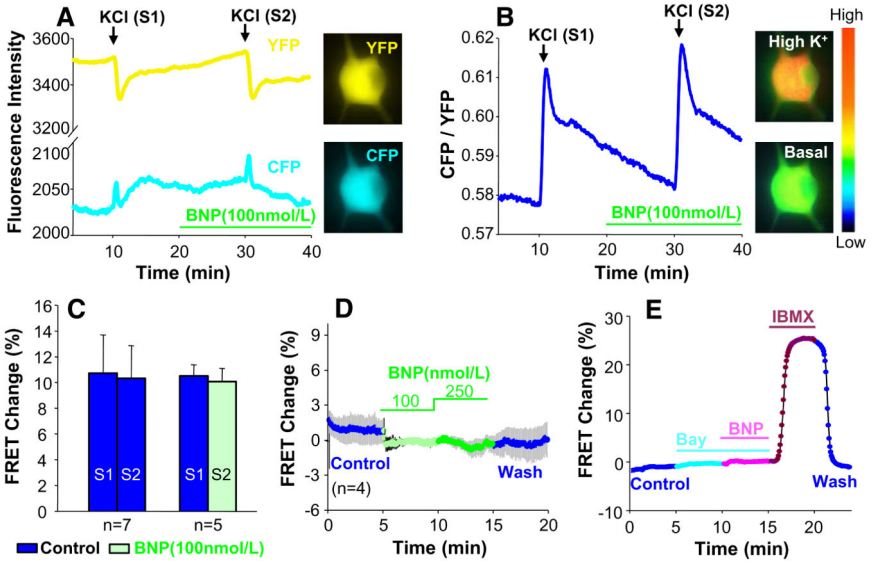

Figure 4.

cAMP measurements in isolated cardiac sympathetic neurons. **A**, Dynamic changes on emission intensity in the cyan fluorescent protein (CFP) channel (cyan) and yellow fluorescent protein (YFP) channel (yellow). Black arrows indicate depolarization of the neuron with 50 mmol/L KCl for 1 minute at 10 minutes (S1) and 30 minutes (S2). B-type natriuretic peptide (BNP) was introduced at 20 minutes. **B**, Representative kinetics of cAMP-induced fluorescence resonance energy transfer (FRET) changes by ratiometric recording of CFP and YFP emission in response to depolarization. **C**, Mean absolute values of FRET change during depolarization in the presence or absence of BNP. **D**, BNP was slightly reduced basal cAMP concentration in cardiac sympathetic neurons (n=4). **E**, Phosphodiesterase 2 inhibitor Bay60-7550 (1 μmol/L) and 100 nmol/L BNP did not significantly affect basal FRET (n=4). Nonspecific inhibition of phosphodiesterases with 3-isobutyl-1-methylxanthine (IBMX, 100 μmol/L) enhanced the intracellular cAMP levels.

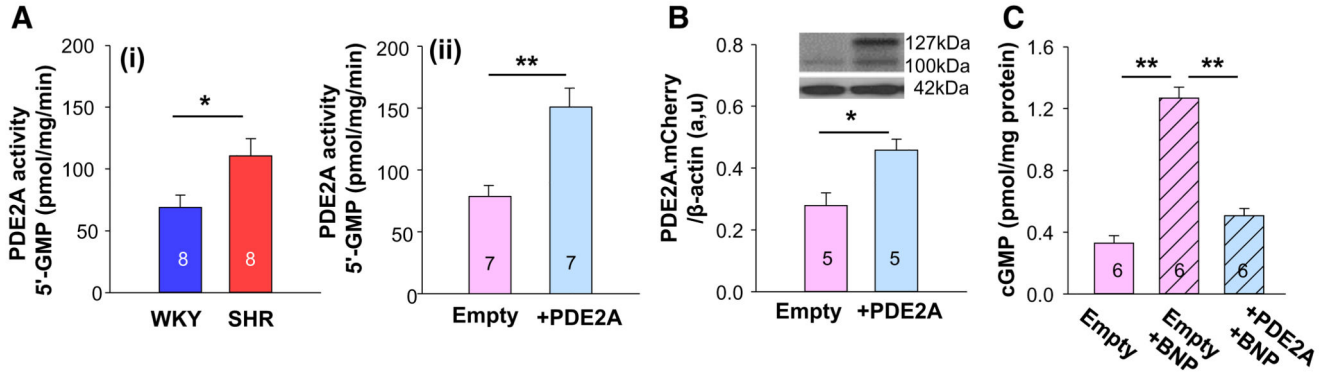

Figure 5.

A, Phosphodiesterase 2 (PDE2) activity in stellate ganglia tissue from prehypertensive spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats (i), normal Sprague Dawley rats transduced with empty and Ad.PDE2A (ii). PDE2 activity was measured by colorimetric assay and defined as the production of 5′-GMP inhibited by 1 μmol/L Bay60-7550. **B**, Western blot showing PDE2A.mCherry expression (127 KDa) in transduced empty and PDE2A stellate ganglia tissue (with anti-PDE2A antibody). Band optical density was normalized to that of β-actin (42 KDa). **C**, Effect of 250 nmol/L B-type natriuretic peptide (BNP) on cGMP concentration in transduced empty and PDE2A stellate ganglia tissue. **P*<0.05, ***P*<0.01, unpaired *t* test.

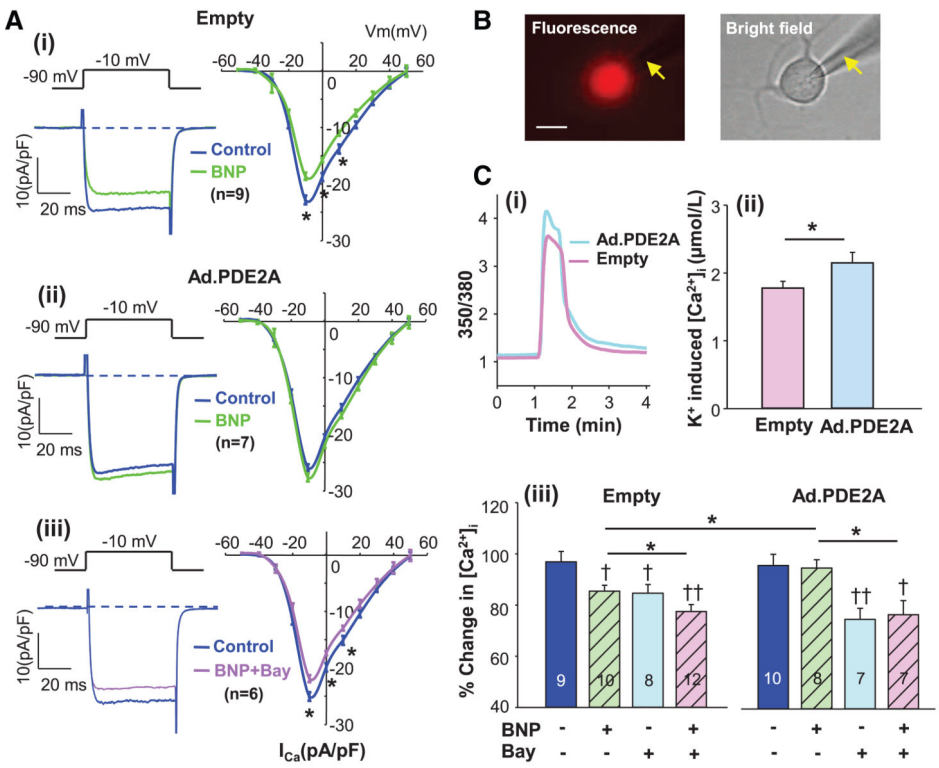

Figure 6.

A, Representative calcium current traces (**left**) and mean current density–voltage relations (**right**) obtained before and after exposure to 100 nmol/L BNP (i and ii) or B-type natriuretic peptide (BNP) with 1 μmol/L Bay60-7550 (iii) in the transduced empty (i) and Ad.PDE2A (ii and iii) cardiac sympathetic neurons. **P*<0.05, paired *t* test. I_{Ca} indicates calcium current; pA, picoampere; and pF, picofarad. **B**, Images of single stellate neurons transfected with empty/Ad.PDE2A after 12 hours for patch clamping. The yellow arrows point to the patch pipette attached to the transduced mCherry (red) neuron. Scale bar represents 20 μm. **C**, Ratio data traces (i) and statistical data (ii) showing 50 mmol/L KCl evoked intracellular calcium transient ($[Ca^{2+}]_i$) in the transduced empty and Ad.PDE2A cardiac sympathetic neurons. (iii) Group data showing KCl evoked peak $[Ca^{2+}]_i$ changes in response to 100 nmol/L BNP and the phosphodiesterase 2 (PDE2) inhibitor Bay60-7550 (1 μmol/L). **P*<0.05, unpaired *t* test; †*P*<0.05, ††*P*<0.01, unpaired *t* test, compared with control within same group.

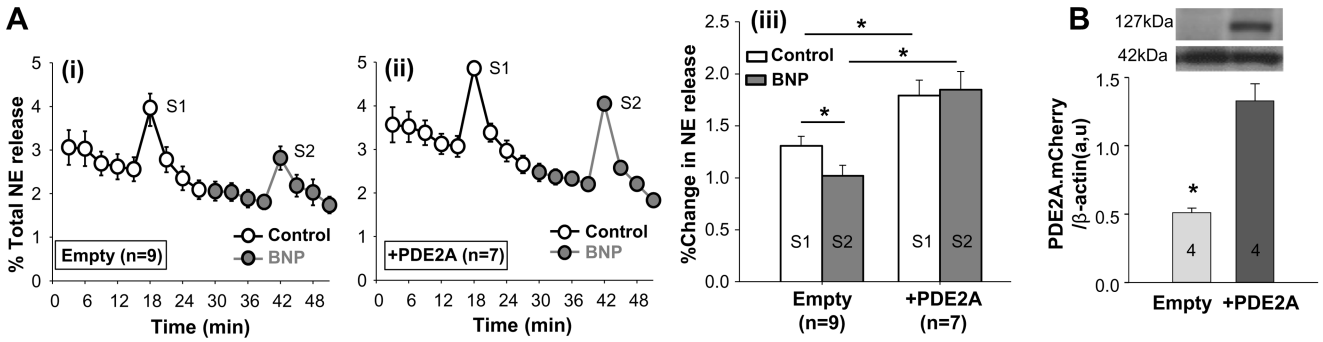

Figure 7.

A, Representative raw data traces (i and ii) and group data (iii) showing [^{3}H]-norepinephrine release during 5-Hz field stimulation from percutaneous gene transfer to right atria with empty virus (i) or Ad.PDE2A (ii). Norepinephrine release was significantly enhanced in the Ad.PDE2A transduced group when compared with the empty control (S1 vs S1; iii). B-type natriuretic peptide (BNP, 250 nmol/L) significantly reduced norepinephrine release (S1 vs S2) in empty (i), but not in transduced Ad.PDE2A group (ii). **P*<0.05, unpaired *t* test. Numbers indicate the number of neurons. **B**, Representative Western blot showing PDE2A.mCherry expression (127KDa) in percutaneous gene transfer to right atria with Ad.PDE2A or empty virus (with antimCherry antibody). Band optical density was normalized to that of β-actin (42KDa) as a loading control. **P*<0.05, unpaired *t* test. Numbers indicate the number of atria. PDE2A indicates phosphodiesterase 2A; and NE, norepinephrine.

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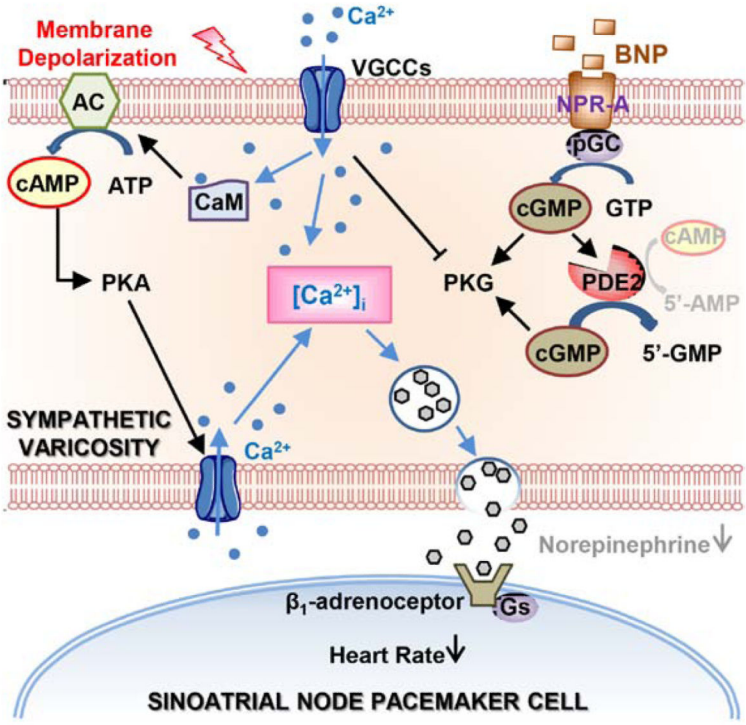

Figure 8.

Diagram illustrating the potential signal transduction pathways mediated by B-type natriuretic peptide (BNP) in depolarized stellate neurons. BNP binds to the natriuretic peptide receptor A (NPR-A) and stimulates particulate guanylyl cyclase (pGC). Newly synthesized cGMP activates phosphodiesterase 2 (PDE2) to degrade cGMP, but not cAMP, and thus limits the increase in protein kinase G (PKG) activity. Moreover, PKG inhibits calcium current by phosphorylating voltage-gated calcium channels (VGCCs) and reduces the intracellular calcium transient. This in turn decreases norepinephrine release from synaptic vesicles and reduces the heart rate response to sympathetic stimulation. In addition, elevated intracellular calcium triggers the activation of adenylate cyclase (AC) via calmodulin (CaM) and produces cAMP. cAMP activates cAMP-dependent protein kinase (PKA), thus phosphorylation of calcium channel.