Effects of ANP and BNP on the generation of respiratory rhythms in brainstem–spinal cord preparation isolated from newborn rats

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ABSTRACT

Natriuretic peptides (NPs) are a family of peptide hormones produced in cardiac muscle cells and consist mainly of three types: atrial NP (ANP), B-type (or brain) NP (BNP), and C-type NP. We herein report the effects of ANP and BNP on central respiratory activity in brainstem–spinal cord preparation isolated from newborn rats. Bath application of these peptides (100 nM) induced a weak transient depression of the respiratory rhythm followed by recovery. Respiratory-related neurons in the rostral ventrolateral medulla showed a tendency for transient hyperpolarization followed by recovery during the application of ANP or BNP. The application of a membrane-permeable cGMP, 8-Br-cGMP (10 or 20 μM), did not induce significant effects on respiratory rhythm, suggesting no involvement of guanylyl cyclase in effects of ANP or BNP. We also examined effects of BNP on respiratory depression induced by the sedative dexmedetomidine, which exerts an inhibitory influence on respiratory rhythm. When pretreated with 50 nM BNP, the inhibitory effect of 100 nM dexmedetomidine was significantly reduced. Our findings suggest that ANP and BNP act as mild excitatory agents with sustained effects on respiratory rhythm after an initial transient depression.

INTRODUCTION

Natriuretic peptides (NPs) are a family of peptide hormones produced in cardiac muscle cells and consist of three major types: atrial NP (ANP), B-type (or brain) NP (BNP), and C-type NP. NPs are involved in cardiovascular regulation and exert natriuretic, diuretic, and vasodilatory functions (Nishikimi et al., 2006; Cao and Yang, 2008; Potter et al., 2009). NP receptors are widely distributed in various tissues, including the central nervous system (CNS) (Imura et al., 1992; Summers and Tang, 1992; Kobayashi et al., 1993; Tang et al., 1993). Indeed, NPs are known to play a role in various CNS functions through the control of synaptic activity: e.g., release and/or uptake of noradrenaline in rat hypothalamus and medulla oblongata (Thiriet et al., 2001), and release of glycine in rat spinal cord sensory circuits (Maeda et al., 2013) (for review, Mahinrad et al., 2016).

Serum BNP levels are known to be elevated in patients with heart failure and are used as a diagnostic biomarker of heart failure in clinical practice (Gaggin and Januzzi, 2013). BNP is mainly secreted by left ventricular myocytes, and the secretion is stimulated by increased cardiac volume and pressure (Boerrigter et al., 2009). In patients with heart failure, the prevalence of sleep apnea syndrome is 50% (Garrigue et al., 2007), and subjects with central sleep apnea syndrome (CSAS) were shown to have high levels of ANP and BNP (Calvin et al., 2011). Therefore, a clinical question is whether diuretic peptides such as BNP may be involved in the cause of CSAS, which is often associated with heart failure (Lanfranchi et al., 2003). However, there have been no studies on the effects of NPs on central re-
The brainstem–spinal cord preparation isolated from newborn rats preserves the neuron networks essential for respiratory rhythm generation and can produce respiratory activity for several hours under in vitro condition (Suzue, 1984; Ballanyi et al., 1999; Katsuki et al., 2021). This preparation has great advantages for pharmacological study of the respiratory center because it can be maintained in an anesthetic-free condition and drugs can be applied by superfusion in known concentrations. Therefore, in the present study, we investigated the effects of ANP and BNP on respiratory activity in isolated brainstem–spinal cord preparation from newborn rats. We also examined the effects of BNP on respiratory depression by dexmedetomidine (Dex), which is used for sedation in the intensive care unit, where patients frequently have cardiovascular diseases (e.g., heart failure or arrhythmia) and show high BNP values.

MATERIALS AND METHODS

Preparation and solutions. The experimental protocols were approved by the Animal Research Committee of Showa University (approval nos. 09049, 02022, 03066) according to Law No. 105 for the care and use of laboratory animals by the Japanese government. All efforts were made to minimize the number of animals used and their suffering.

Brainstem and spinal cord were isolated from Wistar rats on postnatal day 0–3 under deep isoflurane anesthesia. The samples were cut slightly rostral to the anterior inferior cerebellar artery and caudally at the 6–8th cervical cord and were continuously perfused at a rate of 2.5–3 mL/min with artificial cerebrospinal fluid (ACSF) (Suzue, 1984) composed of (in mM): 124 NaCl, 5 KCl, 1.2 KH₂PO₄, 2.4 CaCl₂, 1.3 MgCl₂, 26 NaHCO₃, 30 glucose, equilibrated with 95% O₂ and 5% CO₂, at pH 7.4, at 25–26°C.

Drugs. ANP and BNP (Peptide Institute Inc., Osaka, Japan) were stored at −20°C as a stock solution for histological analysis, and transverse 50-μm slices were cut with a vibrating-blade tissue slicer (PR07; Dosaka EM Co. Ltd., Osaka, Japan). Labeled neurons were photographed using a fluorescence microscope (BX60; Olympus Optical, Tokyo, Japan). The locations of the cell bodies of the recorded neurons were confirmed after staining with NeuroTrace (435/455 blue fluorescence; Molecular Probes/Invitrogen).

Electrophysiology. Respiratory activity was monitored by the fourth cervical ventral root activity (C4), which was amplified through a 0.5 Hz high-pass filter (MEG-5200; Nihon Kohden, Tokyo, Japan). We also recorded the membrane potential of respiratory-related neurons, including pre-inspiratory or inspiratory neurons of the rostral ventrolateral medulla at the level of 0–400 μm caudal to the caudal end of the facial nucleus (Ballanyi et al., 2009; Katsuki et al., 2021). Intracellular recordings were performed using a blind whole-cell patch-clamp method (Onimaru and Homma, 1992; Katsuki et al., 2021), with a high input impedance DC amplifier (CEZ-3100; Nihon Kohden) and by the ventral approach. Electrodes were made by pulling thin-wall borosilicate glass (GC100TF-10; Harvard Apparatus LTD, Kent, UK) while heating. The diameter of the inner tip was 1.2–2.0 μm and the resistance was 4–8 MΩ. The electrodes were filled with a pipette solution (in mM) of 130 K-gluconate, 10 EGTA, 10 HEPES, 2 Na₂-ATP, 1 CaCl₂, 1 MgCl₂, at pH 7.2–7.3 adjusted by KOH. The electrode tip was filled with 0.5% Lucifer Yellow (lithium salt, Sigma-Aldrich) dissolved in the same solution for histological analysis of the recorded cells.

After the experiment, the samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS) at 4°C and stored for histological analysis, and transverse 50-μm slices were cut with a vibrating-blade tissue slicer (PR07; Dosaka EM Co. Ltd., Osaka, Japan). Labeled neurons were photographed using a fluorescence microscope (BX60; Olympus Optical, Tokyo, Japan). The locations of the cell bodies of the recorded neurons were confirmed after staining with NeuroTrace (435/455 blue or 530/615 red fluorescence; Molecular Probes/Invitrogen).

Data analysis. The initial data analysis was performed using the Lab Chart 7 Pro software program (AD Instruments). The burst rate was calculated as an average of 3–5 min. The significance of the values was analyzed using a paired t-test or one-way or two-way ANOVA, followed by a Tukey-Kramer multiple comparison test at a confidence level of P < 0.05 using the GraphPad Prism 6 software program (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

The application of 100 nM ANP in the bath (15 min) induced a weak and transient depression (at 4–7 min) of the respiratory rhythm (C4 burst rate), which was
followed by recovery, even before washing (Fig. 1A). After washout, the rhythm tended to increase further. The bath application of 100 nM BNP (15 min) also induced responses similar to those of ANP (Fig. 1B). Table 1 shows the averaged changes in C4 burst rate to application of 100 nM ANP \((n = 10)\) or BNP \((n = 9)\), which indicate a tendency for transient depression followed by recovery.

We examined membrane potential responses to the application of 100 nM ANP in 5 pre-inspiratory neurons in the rostral ventrolateral medulla. Fig. 2 shows an example of the membrane potential recording from a pre-inspiratory neuron. Fig. 2A demonstrates a slower sweep representation of the membrane potential trajectory (V_m) and C4 nerve activity (C4), and Fig. 2B exhibits faster sweep representations of the traces corresponding to B-a–B-c in A. The application of ANP induced a slight membrane hyperpolarization (B-b) that was followed by a mild depolarization (B-c) during the application of 15 min. The cell was located in the ventrolateral medulla at the level of 400 μm caudal to the caudal end of the facial nucleus (Fig. 2C). Table 2 shows the averaged membrane potentials from 5 neurons, which indicate a tendency for transient hyperpolarization followed by a mild depolarization during ANP application. Next, we examined the membrane potential responses to 100 nM BNP in two pre-inspiratory, three inspiratory, and 1 expiratory neuron. Fig. 3 shows an example of the membrane potential recording from a pre-inspiratory neuron. Fig. 3A demonstrates a slower sweep representation of the membrane potential trajectory (V_m) and C4 nerve activity (C4), and Fig. 3B exhibits faster sweep representations of the traces corresponding to B-a–B-c in A. Membrane potential responses similar to ANP, that is, initial transient hyperpolarization (B-b) followed by depolarization (B-c), were observed with the application of BNP. The cell was located in the ventrolateral medulla at the level of 50 μm caudal to

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**Table 1** Effects of ANP and BNP on respiratory rhythm

<table>
<thead>
<tr>
<th></th>
<th>100 nM ANP ((n = 10)) (bursts/min)</th>
<th>100 nM BNP ((n = 9)) (bursts/min)</th>
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<tbody>
<tr>
<td>Control</td>
<td>6.1 ± 1.9</td>
<td>6.0 ± 1.5</td>
</tr>
<tr>
<td>4–7 min</td>
<td>4.5 ± 1.9</td>
<td>*5.0 ± 1.3</td>
</tr>
<tr>
<td>10–15 min</td>
<td>5.6 ± 1.9</td>
<td>5.8 ± 2.1</td>
</tr>
<tr>
<td>Wash 15 min</td>
<td>6.1 ± 2.5</td>
<td>$6.5 ± 1.8$</td>
</tr>
</tbody>
</table>

ANP or BNP (100 nM) was applied for 15 min. Control: C4 burst rate (bursts/min) prior to application of drugs, 4–7 min: values at 4–7 min after application of drugs, 10–15 min: values at 10–15 min after application of drugs, Wash 15 min: values at 15 min after washout.

* \(P < 0.05\) in comparison to control. \(^*P < 0.05, \^{*}P < 0.01\) in comparison to the values at 4–7 min values (by one-way ANOVA followed by Tukey-Kramer multiple comparison test).
the caudal end of the facial nucleus (Fig. 3C). The averaged membrane potentials from 6 neurons also indicate a tendency for transient hyperpolarization followed by a mild depolarization during BNP application (Table 2).

ANP and BNP mainly act through the activation of guanylyl cyclase, which causes an increase in the intracellular level of cGMP (Cao and Yang, 2008; Chan et al., 2012). Therefore, we examined the effects of 8-Br-cGMP, a membrane-permeable cGMP. The application of 10 μM or 20 μM 8-Br-cGMP did not induce significant effects on respiratory rhythm.
ANP/BNP and respiratory rhythm

Next, we analyzed the effects of BNP on the inhibitory effects of respiratory rhythm by Dex. We previously reported that Dex caused a dose-dependent depression in respiratory rhythm in brainstem–spinal cord preparation (Tsuzawa et al., 2015). In the present study, we chose 100 nM Dex. The application of Dex only induced a gradual depression in respiratory rhythm (Fig. 4, black squares, \( n = 7 \)). In contrast, when 50 nM BNP was applied as pretreatment and co-applied with Dex, the inhibitory effect of Dex was significantly reduced (Fig. 4, red circles, \( n = 7 \)).
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is sensitive to ANP and BNP; NPR-B is highly specific to CNP; and NPR-C binds to all NPs with similar affinities. NPs and their receptors exist not only in the systemic circulation but also in the CNS. NPR-A and NPR-B are receptors coupled to guanylyl cyclase (GC) and possess intrinsic GC activity in the intracellular domain. Although NPR-C lacks a GC catalytic domain and appears to act as a ‘clearance receptor’, several studies have shown that NPR-C could work in intracellular signal transduction pathways such as the adenylyl cyclase (AC)/cAMP or protein kinase C pathway (Anand-Srivastava et al., 1990; Murthy et al., 2000; Rose et al., 2005; Chan et al., 2012). An increase in intracellular cAMP has been reported to facilitate respiratory rhythm in brainstem–spinal cord preparation (Arata et al., 1993). Therefore, the effects of ANP and BNP could be mediated, at least in part, by NPR-C.

NPRs are expressed in glial cells as well as neurons (Cao and Yang, 2008). Astrocytes in the medullary respiratory center were suggested to have significant effects on the generation and/or modulation of respiratory rhythm (Gourine et al., 2010; Okada et al., 2012; Marina et al., 2016; Turovsky et al., 2016). Although it has been suggested that NPRs are expressed in astrocytes of cultured rat spinal cord and brainstem (Hösli et al., 1992), there is no direct evidence of the expression of NPRs in astrocytes of the respiratory center in the medulla. Therefore, although the effects of ANP and BNP in the present study may be at least partially mediated by medullary astrocytes, the expression of NPRs in the medullary respiratory center remains a topic for future study.

Clinical implications
BNP is primarily elevated in heart failure and other circulatory disorders, including coronary artery disease (Lanfranchi et al., 2003; Palazzuoli et al., 2011).

| Table 2 | Effects of ANP and BNP on the membrane potential of respiratory-related neurons in the rostral ventrolateral medulla |
|-----------------|-----------------|-----------------|
| 100 nM ANP (n = 5) | 100 nM BNP (n = 6) |
| **(mV)** | **(mV)** |
| Control | −45.9 ± 2.4 | −47.7 ± 4.4 |
| 4–6 min | −48.0 ± 2.9 | −48.6 ± 4.2 |
| 15 min | −43.9 ± 2.3 | −46.0 ± 3.6 |

ANP or BNP (100 nM) was applied for 15 min. Control: membrane potentials prior to application of drugs, 4–6 min: values at 4–6 min after application of drugs, 15 min: values at 15 min after application of drugs.

$P < 0.05$, $P < 0.01$ in comparison to the values of 4–6 min (by one-way ANOVA followed by Tukey-Kramer multiple comparison test).

| Table 3 | Effects of 8-Br-cGMP on respiratory rhythm |
|-----------------|-----------------|-----------------|
| 10 μM 8-Br-cGMP (n = 8) | 20 μM 8-Br-cGMP (n = 5) |
| **(bursts/min)** | **(bursts/min)** |
| Control | 5.7 ± 1.8 | 5.4 ± 1.6 |
| cGMP 15 min | 5.6 ± 1.9 | 5.7 ± 1.6 |
| Wash 15 min | 5.4 ± 1.8 | 5.6 ± 1.9 |
| Wash 30 min | 5.7 ± 2.2 | 5.9 ± 2.2 |

8-Br-cGMP (10 or 20 μM) was applied for 15 min. Control: C4 burst rate (bursts/min) prior to application of drugs, cGMP 15 min: values at 15 min after application of drugs, Wash 15 min: values at 15 min after washout, Wash 30 min: values at 30 min after washout.

No values were significantly different (by one-way ANOVA).

DISCUSSION

The application of ANP and BNP induced mild effects on respiratory activity in the in vitro brainstem–spinal cord preparation of newborn rats: weak transient depression followed by recovery or significant enhancement of the respiratory rhythm. The changes in the respiratory neuron membrane potential in the rostral ventrolateral medulla were consistent with the changes in respiratory rhythm (i.e., initial slight hyperpolarization followed by depolarization). These results suggested that ANP and BNP have mild excitatory effects on respiratory rhythm after initial transient depression. This view is consistent with the findings that the inhibitory effect of Dex was reduced by pretreatment with BNP. The effects of ANP and BNP were not simply attributable to an intracellular increase in cGMP, because 8-Br-cGMP did not induce significant effects on the respiratory rhythm.

Cellular mechanisms of the effects of ANP and BNP

Three classes of receptors for NPs have been characterized (for review, Cao and Yang, 2008): NPR-A
ANP/BNP and respiratory rhythm

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In practice, BNP is recommended as a diagnostic marker for heart failure (McCullough et al., 2002), and its value is associated with the severity and prognosis of heart failure (Doust et al., 2005). ANP was also considered to have similar properties in the early search for heart failure, but it is not currently used as a biomarker (Jarolim, 2006). The incidence of CSAS is higher in patients with heart failure (Lanfranchi et al., 2003), and ANP and BNP levels are elevated in CSAS (Calvin et al., 2011). These findings imply that CSAS and BNP levels are closely related. Although it is not clear whether systemic BNP (or ANP) directly acts on the respiratory center in the medulla through the blood-brain barrier, the possibility of an interaction between central and systemic NPs has been proposed (Mahinrad et al., 2016). It should be noted that in the present study, however, the drugs were directly applied by superfusion to the medulla and spinal cord and could reach into the tissue not through the blood-brain barrier. Our experiments revealed that BNP induced transient respiratory depression. However, present results did not support that BNP could be involved in respiratory depression in CSAS because transient depression was followed by sustained excitatory effects on respiratory rhythm in our experimental condition. In line with this view, the inhibitory effect of Dex was reduced by pretreatment with BNP. Thus, similar to the cardioprotective secretion of NPs, ANP and BNP may have a protective effect on apnea complicated by heart failure, although the results from experiments with newborn rat in vitro preparations cannot simply be expanded to understand the effects of substances administered in adult in vivo experiments or in the human clinical setting.

In conclusion, ANP and BNP were shown to exert transient inhibitory effects followed by sustained excitatory effects on the respiratory center. The biological significance of these effects on respiration is unknown, but they may protect against central apnea and require further study.

CONFLICT OF INTEREST
The authors declare no conflicts of interest in association with the present study.

Acknowledgments
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