Guanylyl Cyclase Receptors Mediate Cardiopulmonary Vagal Reflex Actions of ANP

Colleen J. Thomas, Robyn L. Woods

Abstract—Atrial natriuretic peptide (ANP) potentiates vagal cardiopulmonary reflexes due to chemosensory (Bezold-Jarisch [B-J] reflex) or mechanosensory (ramp baroreflex) activation. The ANP receptor mediating these actions is unknown. We examined the role of particulate guanylyl-cyclase (pGC) receptors in ANP-induced enhancement of cardiopulmonary vagal reflexes. Cardiopulmonary baroreceptor reflex function was assessed by bradycardic responses to ramp blood pressure rises after rapid intravenous methoxamine (100 μg/kg bolus dose). The B-J reflex was evoked by 3 intravenous doses of serotonin (1 to 10 μg/kg). In conscious, chronically instrumented rats (n=9), these tests were performed on each animal during randomized infusions of rat ANP (150 ng/kg per minute IV), saline (270 μL/h IV), the pGC receptor antagonist HS-142-1 (3 mg/kg IV), or combined HS-142-1+ANP treatment. HS-142-1 alone attenuated normal B-J reflex (by 33±8%, P<0.05) but not ramp baroreflex responses. As we showed previously, ANP enhanced baroreflex and B-J reflex bradycardia (by ≈140% and ≈30%, respectively, P<0.05), compared with saline infusion. These ANP effects were completely blocked by HS-142-1, demonstrating that the cardiopulmonary vagal reflex actions of ANP occurred through pGC natriuretic peptide receptors. Additionally, we have provided evidence for the first time that pGC natriuretic peptide receptors are essential for the full expression of the B-J reflex but not for that of cardiopulmonary vagal baroreflexes. This tonic interaction between pGC natriuretic peptide receptors and cardiopulmonary chemosensitive receptors may be important during pathophysiological activation of B-J reflex, such as with myocardial infarction. (Hypertension. 2003;41:279-285.)

Key Words: atrial natriuretic factor ■ autonomic nervous system ■ baroreflex ■ bradycardia ■ cyclic GMP ■ natriuretic peptides

In previous studies, we1–5 and others (for reviews see Volpe and Clemo et al7) presented evidence that the cardiac natriuretic peptides play a role in reflex control of heart rate (HR). We demonstrated that all 3 natriuretic peptides (atrial natriuretic peptide [ANP], B-type natriuretic peptide [BNP] and C-type natriuretic peptide [CNP]) sensitize cardiopulmonary vagal reflexes to slow HR.4,5 There are 3 known natriuretic peptide receptor subtypes: A (NP_A), B (NP_B), and C (NP_C).8 Most of the well-known biological actions, such as vasodilation and natriuresis, of the natriuretic peptides occur subsequent to stimulating the production of the second-messenger cyclic guanosine 3’ 5’-monophosphate (cGMP), through particulate guanylyl-cyclase (pGC)-coupled receptors (the NP_A and NP_B receptors). The NP_C receptor fulfills the role of a clearance receptor for the 3 natriuretic peptides.8 In addition, while not having guanylyl-cyclase activity, this receptor has been reported to influence 3 signal-transducing systems: phospholipase C, adenylyl cyclase, and calcium channels.9 The NP_C receptor is proposed to be a physiological regulator of activities such as adrenergic transmission, renin and progesterone secretion, and platelet aggregation.9

Although there is a growing body of literature linking natriuretic peptide receptors to the more widely known cardiovascular and renal actions of ANP, BNP, and CNP,10–15 the nature of the natriuretic peptide receptors involved in the HR reflex activities of these hormones has not been studied or reported in the literature. Since BNP and CNP share with ANP the ability to enhance reflex bradycardic responses,4,5 a common receptor may mediate these effects. In the present studies, the contribution from the guanylyl cyclase receptors to the cardiopulmonary vagal baroreflex and chemoreflex (Bezold-Jarisch [B-J] actions of ANP was investigated. HS-142-1 was used to selectively antagonize the NP_A+NP_B guanylyl cyclase–coupled (pGC) receptors.16 HS-142-1 is a polysaccharide microbial product isolated from the culture broth of Aureobasidium species.16 HS-142-1 blocks the vasodilator and renal effects of exogenously administered ANP and BNP and also reverses the changes resulting from augmented endogenous production of ANP and BNP.10,17,18 Although HS-142-1 can exclude a role for the non–guanylyl cyclase receptor NP_C,16 this compound does not distinguish between subclasses of the pGC natriuretic peptide receptors. To discriminate between the roles of the NP_A and NP_B receptors, A71915 was used to selectively block NP_A receptors.19,20 A71915 is a structural analog of ANP19 with a...
binding affinity to rat NPA receptors only 22 times less potent than native rat ANP (1–28).21

Methods

Experimental Protocol
Experiments were performed at least 1 week after instrumentation in conscious, unrestrained rats. Mostly the animals had 2 experimental days; however, 3 animals had 4 experimental days since they participated in tests of both natriuretic peptide receptor blockers. Each experimental day involved testing HR reflex responses in the presence of 2 different treatments. A completely randomized protocol was not possible because a treatment without blockade (eg, saline or ANP) could not follow blocker treatment due to the potentially long-lasting nature of their actions. Animals with more than 2 experimental days had a rest day between experiments. Rats receiving HS-142-1 on the first experimental day also had a rest day before completing the second day of testing to eliminate any possible carryover effects of HS-142-1. As described previously,6 phasic blood pressure and HR were recorded continuously with the use of a computerized data acquisition system. A 60-minute period of acclimatization in each rat preceded assessment of cardiopulmonary baroreflexes.

Treatments
In all rats, HR reflex responses to activation of cardiopulmonary mechanoreceptors (ramp method) and chemoreceptors (B-J reflex) were measured in the presence of alternate infusions of 0.9% saline (270 μL/h IV) or rat ANP (150 ng/kg per minute IV; Phoenix Pharmaceuticals). In one group of 9 rats, the pGc natriuretic peptide receptor antagonist HS-142-1 (3 mg/kg IV bolus every 30 minutes; a gift from Kyowa Hakko Kogyo Co Ltd, Tokyo, Japan) with or without ANP was used. To distinguish between contributions from NPA and NPA receptors, the “selective” NPA receptor antagonist A71915 (10 μg/kg IV bolus injection followed by 10 μg/kg per minute IV infusion, Abbot Laboratories) with or without ANP was used in 7 rats.

HR Reflex Techniques
Cardiopulmonary baroreceptor (“ramp”) and chemoreceptor (B-J reflex) HR reflexes were assessed by methodology we previously described.2 For the “ramp” method, rats were given 3 replicate fast injections of methoxamine (~100 μg/kg IV doses; Sigma), resulting in rapid increases in MAP and reflex falls in HR. Linear regression analysis was applied to the data, with a 0.5-second delay fitting HR responses to MAP changes.2 Baroreflex sensitivity was determined from the slope of the relation between these 2 variables. The average of the best 2 or 3 ramps (based on comparable rate of blood pressure change) was taken as ramp gain or sensitivity in each case for further analysis. The B-J reflex was activated by bolus injections of serotonin (5-hydroxytryptamine (5-HT), creatinine sulfate complex, Sigma Chemical Co, 1 to 14 μg/kg IV), producing dose-dependent HR reductions. Although there was variability between animals in responses to 5-HT, identical doses of 5-HT were used for all treatments within each rat. The same 3 doses of 5-HT in each animal were selected for analysis, covering a range of responsiveness from just above threshold, to intermediate, to maximal.

Statistical Analysis
Except where indicated, values are mean±SEM. Data for B-J experiments were analyzed by 3-factor ANOVA, with the use of SigmaStat (Version 2.03, SPSS Inc). The 3 factors were rat, treatment, and dose. Significant effects of treatments were determined by Bonferroni i test with adjustment for multiple comparisons. The ramp sensitivities were analyzed by 2-way ANOVA with factors being rat and treatment. Orthogonal partitioning of the sums of squares was used to determine significant effects of treatment with Bonferroni adjustment for multiple comparisons.2 Significant effects were taken at the level of \( P<0.05 \).

Results

Effect of HS-142-1
In the group of 9 rats tested for the effects of HS-142-1, average MAP was 116±2 mm Hg and HR was 344±10 bpm during saline infusion. Compared with their own preinfusion control levels, neither HS-142-1 alone nor ANP alone significantly altered resting MAP or HR (Table 1). Combined HS-142-1+ANP treatment, however, caused a small but significant (\( P<0.05 \)) fall in resting MAP (Table 1); resting HR also tended to fall with combined treatment, but these changes did not reach significance (Table 1). HS-142-1 treatment alone had no significant effect on ramp baroreflex gain compared with saline (difference of 0.23±0.41 bpm/mm Hg; Figure 2A and Table 1). ANP, alone, enhanced ramp gain by \( \approx 2.4\)-fold (\( P<0.05 \); Figure 2 and Table 1), and this facilitation was completely absent in the presence of HS-142-1 (Figure 2A and Table 1). The average rate of rise in MAP and the blood pressure range achieved during the phenylephrine ramps were similar across treatments (Table 1).

In the presence of saline, 5-HT decreased resting HR by 14±3, 108±28, and 209±20 bpm in response to low, medium, and high doses, respectively (Figure 2A). The average reflex bradycardia over all doses was 110±24 bpm (Figure 2B and Table 1). The mean doses of 5-HT into the jugular vein to achieve these changes were 2.9±0.6, 6.5±0.5, and 10.3±0.7 μg/kg. Mean changes in MAP recorded at the time of maximum bradycardia to low, medium, and high doses were 2±1, 5±1, and 30±6 mm Hg, respectively (Figure 3A), with average of 13±4 mm Hg over all the doses (Figure 3B and Table 1). HS-142-1 given alone significantly reduced mean chemoreflex bradycardia to 5-HT compared with saline infusion (by 38±11 bpm, \( P<0.05 \); Figure 2A and Table 1). In addition, HS-142-1 given alone significantly reduced the average hypotensive response to 5-HT compared with saline infusion (by 8±2 mm Hg, \( P<0.05 \); Figure 2B and Table 1). As shown previously,2,3,23 ANP infusion significantly augmented the B-J reflex bradycardia (average of 143±24 bpm over all doses; \( P<0.05 \)), although the accompanying hypotension was not significantly augmented (Figure 2B and Table 1). In the presence of HS-142-1, the ANP enhancement of the B-J reflex was completely prevented. Reflex bradycardia and hypotension to 5-HT in the presence of combined HS-142-1+ANP infusions were not significantly different from control 5-HT responses with saline infusion (Figures 2B and 3B and Table 1).
Effect of A71915

We found that A71915 behaved with partial agonist action since, when given alone, it potentiated the ramp baroreflex (by \(\approx 87\%\), Table 2) and B-J reflex (by \(\approx 45\%\), Table 2), just like ANP did when given alone (by \(\approx 70\%\) and \(\approx 25\%\), respectively, Table 2). Reflex responses with combined ANP and A71915 (10 µg/kg per minute) infusions were not different from responses evoked with either of these agents alone (Table 2). Since either peptide (ANP or A71915) may have caused close to maximum facilitation of the reflexes, we could not determine whether A71915 blocked any further effects of ANP. A 10-fold-lower dose of A71915 in 3 rats still showed intrinsic activity and did not block ANP-induced facilitation of cardiopulmonary vagal reflexes. We concluded that this lower dose of A71915 was insufficient to block ANP actions and yet still had agonist properties. Because of this complication of agonist properties of A71915, even at the lower dose, these experiments were not continued in more animals.

Discussion

A major finding of these studies was that HS-142-1 blocked the ability of ANP to enhance reflex bradycardic responses, demonstrating a particulate guanylyl cyclase-coupled (pGC) receptor mechanism for ANP effects on cardiopulmonary vagal reflexes. This was true for both mechanoreceptor and chemoreceptor reflexes. The present study also introduces the novel concept that these pGC natriuretic peptide receptors may be tonically active and demonstrates that this tonic activity is essential for full expression of 5-HT\(_3\)-activated cardiopulmonary chemoreflexes.

What is the location of the pGC natriuretic peptide receptors that potentiate cardiopulmonary reflexes? The possibilities include sites influencing afferent, efferent, or central pathways. In the case of central pathways, we only need to consider actions at sites accessible by blood-borne peptide or receptor antagonist, that is, the sensory circumventricular organs (area postrema, subfornical organ, and organum vasculosum of the lamina terminalis\(^{23}\)), which lack a blood-brain barrier. In particular, the area postrema has natriuretic peptide binding sites with pharmacological characteristics of the NP\(_A\) and NP\(_B\) but not the NP\(_C\) receptor subtypes.\(^{25}\) Commonalities in central connections of cardiopulmonary mechanosensitive and chemosensitive afferent pathways make this an interesting possibility, although there is no evidence yet either for or against it.

Although it is true that both mechanoreceptor- and chemoreceptor-driven cardiopulmonary reflexes may be ex-
Expected to inhibit sympathetic activity, heart rate itself responds only very sluggishly to sympathoinhibition. The rapid bradycardias (1 second rise time) measured in the present experiments were therefore parasympathetically mediated. Some evidence suggests that ANP could act by enhancing cardiac vagal efferent neurotransmission. Atchison and Ackermann found that ANP, infused into rats at twice the dose used in the present study, potentiated the bradycardia in response to direct stimulation of the efferent vagus. On the other hand, Herring et al. found no effect of even high doses of ANP on efferent vagal transmission to guinea pig hearts in vitro. It remains possible that ANP could act in vivo to antagonize some factor not present in vitro, such as tonic α₁ presynaptic modulation, perhaps at the level of the cardiac ganglia. When the direct influence of sympathetic nerves was tested in vivo, however, no effect of ANP action on vagal efferent transmission could be detected. Any role for sympathetic nerves in the action of ANP on cardiopulmonary heart rate reflexes thus seems unlikely.

Against the view that ANP acts on efferent vagal transmission, at least in the lower doses used in the present study, is the finding that ANP does not potentiate arterial baroreflex bradycardia (measured by the steady-state method). Nevertheless, we cannot exclude the possibility that ANP has its action by selective potentiation of efferent transmission in a subset of parasympathetic neurons that are not accessed by arterial baroreceptors (eg, C-fiber cardiac vagal efferents). Despite this, we consider it more likely that ANP’s main action is through pGC natriuretic peptide receptors on, or closely associated with, cardiac vagal afferent terminals. This latter hypothesis would most economically explain why ANP acts to enhance cardiopulmonary mechanoreceptor and chemoreceptor reflexes but not those from arterial receptors and unifies it with the well-accepted role of cardiopulmonary afferents in the sympathoinhibitory actions of ANP. The pGC natriuretic peptide receptors need not be on the afferent terminals themselves. They could be located in cardiac tissue close to the vagal afferent terminals and release some"
intermediary factor such as nitric oxide or prostaglandins to enhance afferent excitability. Although pGC natriuretic peptide receptors have been found recently in human coronary artery tissue (the most effective access route to afferents triggering the B-J reflex), it is not known whether there are pGC natriuretic peptide on cardiac sensory afferents themselves. All 3 natriuretic peptide receptor subtypes are present in atrial and ventricular tissue of rat and human hearts as well as in isolated ventricular cardiomyocytes and fibroblasts, but there is no evidence to date to show the existence of pGC natriuretic peptide receptors on cardiac autonomic nerves.

A link between ANP, cardiac 5-HT₃ receptors, and cardiopulmonary vagal reflexes has been suggested by other investigators. Deliva and Ackermann reported that the B-J-like reflex effects of bolus intravenous doses of ANP were reduced when Ondansetron, a 5-HT₃ receptor antagonist, was administered either into the pericardium or intravenously. These workers proposed that blood-borne ANP, or a secondary substance released by ANP, acts on 5-HT₃ receptors located on chemosensory afferent fibers in the myocardium to cause bradycardia and sympathoinhibition. This proposal is consistent with our present findings that 5-HT₃ sensitivity and pGC natriuretic peptide receptor activity are closely linked. It is possible that pGC natriuretic peptide receptors and 5-HT₃ receptors are in close proximity to, and may communicate directly with, each other on the chemosensory afferents themselves. (Nearly all vagal afferent neurons are known to express 5-HT₃ receptors. Alternatively, perhaps coronary vascular pGC natriuretic peptide receptors talk indirectly to 5-HT₃ chemosensory afferents. Regardless of the means by which cardiopulmonary chemosensory pathways and pGC natriuretic peptide receptors communicate, our results take the role of natriuretic peptides firmly out of the pharmacological and into the physiological sphere. This may mean that only modest activation of the chemosensory afferents, for example by low levels of serotonin that might be released from platelets, is required for an effective B-J reflex response, provided there is tonic sensitization from pGC natriuretic peptide receptors.

It is a novel concept that tonically active pGC natriuretic peptide receptors, presumably driven by endogenous ANP, BNP, or CNP, modulate cardiopulmonary vagal reflexes. Since we have shown previously that exogenous ANP and CNP enhance the B-J reflex, the inhibitory effect of HS-142-1 alone on cardiopulmonary chemoreflex function may be attributable to any one of the endogenous natriuretic peptides. Our data show apparently selective tonic effects of HS-142-1 on cardiopulmonary chemosensory reflexes, yet we cannot completely eliminate the possibility that pGC natriuretic peptide receptors also have a tonic effect on mechanoreceptors. Indeed, HS-142-1 blocked the cardiopulmonary baroreflex–sensitizing actions of ANP as well as it blocked the potentiating effects of ANP on the B-J reflex. It may be that our stimulus to the cardiopulmonary mechanoreceptors (rapid rise in blood pressure) is not as powerful a stimulus as serotonin is to 5-HT₃ receptors on the chemosensory afferents, and therefore any tonic influence of pGC natriuretic peptide receptors on the cardiopulmonary mechanoreceptor pathways is more difficult to measure.

Since there remained no residual activity of ANP in the presence of HS-142-1 on either cardiopulmonary vagal reflex, it seems unlikely that the NP₅ receptor is involved in the ANP response. Our previous results from comparative studies with the 3 natriuretic peptides indicated that the NP₅ receptor was the most likely candidate receptor. The potency ratio for the natriuretic peptides was BNP slightly ANP CNP in potentiating either the cardiopulmonary baroreflex in rats or the B-J reflex in sheep. This potency ratio is not consistent with the primary receptor being the NP₅. Nevertheless, we tried to confirm the role of the NP₅ receptor with the so-called selective NP₅ receptor antagonist, A71915. It was disappointing to find strong

### Table 2: Effect of A71915 + ANP Treatment on Baseline Hemodynamics and on Cardiac Baroreceptor and B-J Reflex Responses in Conscious Munich-Wistar Rats (n = 7)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline</th>
<th>ANP</th>
<th>A71915</th>
<th>A71915 + ANP</th>
<th>SED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline values</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change in resting MAP (after 20-min treatment), mm Hg</td>
<td>0</td>
<td>-3</td>
<td>-4</td>
<td>-13</td>
<td>±5</td>
</tr>
<tr>
<td>Change in resting HR (after 20-min treatment), bpm</td>
<td>10</td>
<td>10</td>
<td>-8</td>
<td>-13</td>
<td>±16</td>
</tr>
<tr>
<td>Ramp method</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gain, bpm/mm Hg</td>
<td>-1.93</td>
<td>-3.29*</td>
<td>-3.61*</td>
<td>-3.69*</td>
<td>±0.29</td>
</tr>
<tr>
<td>MAP range, mm Hg</td>
<td>45.1</td>
<td>44.9</td>
<td>44.4</td>
<td>43.6</td>
<td>±1.0</td>
</tr>
<tr>
<td>Rate of change in MAP, mm Hg/s</td>
<td>26</td>
<td>24</td>
<td>24</td>
<td>21</td>
<td>±2</td>
</tr>
<tr>
<td>Bezold-Jarisch reflex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change in MAP, mm Hg</td>
<td>-9</td>
<td>-10</td>
<td>-13</td>
<td>-13</td>
<td>±4</td>
</tr>
<tr>
<td>Change in HR, bpm</td>
<td>-105</td>
<td>-131*</td>
<td>-153*</td>
<td>-143*</td>
<td>±22</td>
</tr>
</tbody>
</table>

Baseline hemodynamics, baroreflex parameters from rapid ramp infusions of methoxamine, and reflex responses to 5-HT in conscious Munich-Wistar rats with saline or ANP (150 ng/kg per minute IV) infusion or A71915 + ANP treatment. Baseline values refer to the change in resting mean arterial pressure (MAP) or heart rate (HR) after 20-minute treatment compared with pretreatment level. For all parameters, values are means ± SED, where SED is an estimate of within-animal variance for comparison between all treatments.

*Significant difference from saline, P < 0.05.
intrinsic activity of A71915 on both cardiopulmonary vagal reflexes, which made any actions that A71915 might have had as an antagonist impossible to interpret. An ANP-like, or partial agonist, action of A71915 has been described for effects on the kidney37,38 and on cardiomyocytes (Rosenkranz, Howard Florey Institute, recent unpublished observations) but not, for example, in splenic vessels20 or in vas deferens.39 Although we could see no further effect of ANP in the absence of A71915, we could not conclude that A71915 blocked the action of ANP. It seems much more likely that the intrinsic activity of A71915 near-maximally potentiated vagal reflex activity so that no further effect of ANP could be seen.

In summary, HS-142-1 completely prevented the sensitizing action of ANP on cardiopulmonary vagal reflexes to enhance reflex bradycardia from either chemosensory or mechanosensory activation. It appears, therefore, that the guanylyl cyclase natriuretic peptide receptors (NP B and/or NP C) and not the NP A receptor mediate the ANP action on HR reflex responses. The natriuretic peptide receptors in the cardiopulmonary chemosensory pathway are tonically active, and this activity is required for full expression of the B-J reflex.

Perspectives
Our results with HS-142-1, showing that pGC natriuretic peptide receptors play a crucial role in the B-J reflex, open new possibilities for manipulating natriuretic peptides and/or their receptors to treat cardiovascular diseases. The B-J reflex is activated as a cardioprotective reflex under a range of conditions such as with myocardial ischemia or infarction,40 during thrombolytic therapy,41 and during syncope.41 It is possible that activation of the B-J reflex tips the sympathovagal balance toward the parasympathetic, thereby reducing the occurrence of tachyarrhythmias, improving coronary perfusion and aiding recovery from acute cardiac ischemia. Under conditions in which pGC natriuretic peptide receptors are reduced, such as in cardiac hypertrophy42 and in atrial fibrillation,43 effectiveness of the B-J reflex activation may be compromised, contributing to declining cardiac function.

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References


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