Chronotropic Stimulation: A Primary Effector for Release of Atrial Natriuretic Factor

Glenda E. Bilder, Peter K. S. Siegl, Timothy L. Schofield, and Paul A. Friedman

Release of atrial natriuretic factor (ANF) following an elevation in heart rate is thought to be mediated primarily by a change in atrial stretch. To evaluate the direct effect of chronotropic stimulation on ANF release, isolated rat left atria were electrically paced (1–9 Hz) at constant resting tension (0.5–4 g), and the amount of immunoreactive ANF (IRANF) released at each frequency and tension was quantitated with a sensitive radioimmunoassay. Our results show that at controlled resting tensions greater than 1 g, chronotropic stimulation increased IRANF secretion in a manner dependent on the pacing frequency; rapid atrial rates (e.g., 8 and 9 Hz) were necessary to release ANF at tensions of 1 g or less. Resting tension influenced the magnitude of the secretory response to electrical stimulation. Release of IRANF with contraction frequency was transient in nature and, at high frequencies, was associated with a decrease in developed (systolic) tension in accordance with the negative force-frequency relation inherent in the rat heart. When evaluated at a single diastolic tension and pacing frequency, IRANF release was positively correlated with systolic tension. ANF released under in vitro conditions was approximately 3,000 Da, in agreement with the size of the physiologically circulating form. In atria from reserpinized rats, evidence for involvement of catecholamines in chronotropic-stimulated ANF release was suggested. The presence of lidocaine (5×10^-4 M) had no effect on rate-induced ANF secretion. Therefore, chronotropic stimulation releases ANF independently of changes in atrial stretch. The magnitude of this response depends on a combination of pacing frequency and diastolic tension. Catecholamine release and sodium transport through channels sensitive to a local anesthetic appear to play a minor role in rate-dependent ANF release in vitro.

(Circulation Research 1989;64:799–805)

The granules of cardiac atria contain atrial natriuretic factor (ANF), a secretory protein that, by virtue of its renal and cardiovascular effects, is purported to be a hormonal regulator of extracellular fluid volume.

Release of ANF appears to be influenced by the degree of atrial distension. In addition to atrial stretch, the marked elevation in plasma ANF following episodes of paroxysmal tachycardia in humans, intracardiac pacing in humans and animals, and increased ANF secretion with repetitive atrial stretch or electrical stimulation in vitro suggest that heart rate may be an additional stimulus for ANF release. Because changes in atrial pressure (distension) are known to accompany alterations in heart rate in vivo and because developed tension is influenced by changes in rate in vitro, results of previous studies associating contraction frequency with ANF release have been unable to provide convincing evidence for a direct effect of heart rate on ANF release.

The purpose of these experiments, therefore, was to determine whether atrial rate can influence ANF secretion independently of changes in resting (diastolic) tension. Rat atria were used for these experiments because, in this species, developed (systolic) tension is inversely related to contraction frequency. Since diastolic tension influences ANF release, this preparation allowed us to measure ANF release due to increases in atrial rate without concomitant increases in diastolic tension. The relation between rate-induced ANF release and developed tension was also analyzed. Preliminary studies were initiated into the cellular mechanism by which chronotropic stimulation increased ANF release at a constant diastolic tension.

Our results indicate that chronotropic stimulation releases ANF by a mechanism independent of subsequent changes in either diastolic or systolic tension. The mechanism of this type of release does not appear to depend to any great extent on catecholamines or sodium conductance through local anesthetic receptor sensitive channels.

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Received April 5, 1988; accepted September 28, 1988.
Materials and Methods

Tissue Bath Preparation of Left Atria

Sprague-Dawley male rats (200–250 g) were killed by cervical dislocation, and hearts were rapidly removed. Left atria were dissected free, mounted on a Plexiglas tissue holder, and suspended in a 10-ml water jacketed (37°C) tissue bath containing Krebs-Henseleit solution, pH 7.4, gassed with 95% O₂-5% CO₂. The contents of the physiological solution were as follows (mM): NaCl 117, KCl 4.8, MgSO₄ 1.2, CaCl₂ 2.0, K₂HPO₄ 1.2, NaHCO₃ 30.6, dextrose 11.1, and aprotinin (Sigma Chemical Co, St. Louis, Missouri), 50 mg/l. Tension was recorded by a string connecting the atria to an isometric force transducer (Statham-Gould UC3, Gould Inc, Cleveland, Ohio) and a Grass Model 7 polygraph (Grass Instruments, Quincy, Massachusetts). Atria were equilibrated for a period of 30–40 minutes before experimentation.

Electrical Stimulation

Left atria were electrically paced at frequencies of 1–9 Hz with square-wave pulses of 1.0 msec duration, at suprathereshold voltage. At each stimulation frequency, the resting tension was rigorously controlled and maintained by manual vernier adjustment. Fluctuations in resting tension throughout the experiment were less than 1%. Developed (systolic) tension, measured as peak tension minus resting tension, was not constant since both rate and diastolic length influence developed tension.

Immunoreactive ANF

The amount of immunoreactive ANF (IRANF) released in response to a change in resting tension or pacing frequency was assayed by a sensitive radioimmunoassay (RIA) using anti-a-atrial natriuretic polypeptide serum (Peninsula Labs, Inc, Belmont, California). The IC₅₀ of the assay was 31 pg/tube with intra-assay and interassay variability of 4.4% and 5.2%, respectively. IRANF was measured in a 1-ml aliquot during two consecutive 10-minute periods following a change in resting tension or contraction frequency. Samples were boiled for 3 minutes, lyophilized, and stored (−20°C) until assay within 1 week of the experiment. Following these procedures, recovery of synthetic ANF added to the bath and removed after 3 hours of incubation was 99%.

Molecular Weight of Released ANF

ANF secreted into the tissue bath was size separated on a TSK Spiro-gel 2000 SW (7.5 mm i.d. × 60 cm) as described previously. Fractions (1 ml) were lyophilized, reconstituted with RIA buffer, and assayed as above.

Lactic Dehydrogenase Assay

Cellular damage was assessed by lactic dehydrogenase (LDH) release into the tissue bath determined according to a previous method.

Experimental Protocols

The amount of IRANF secreted in response to a change in resting tension was first measured in the unpaced atria. This amount of IRANF, a composite of basal and stretch-induced ANF release, was subtracted from ANF secreted with pacing at the same resting tension. To determine the effect of chronotropic stimulation on ANF release, independent of a change in resting tension, atria were electrically paced at various rates (1, 2, 5, 6, 8, or 9 Hz) with a fixed resting tension of 0.5, 1, 2, 3, or 4 g. No more than three different stimulation frequencies and resting tensions were applied to any one atria. Developed tension was not fixed. Each period of stimulation lasted 20 minutes. IRANF released into the bath was determined at 10 and 20 minutes after a change in atrial rate. After completion of a frequency-release determination at a single resting tension, and before beginning experimentation at a second resting tension, atria were reequilibrated for 45 minutes at zero tension.

The role of endogenous catecholamines in rate-stimulated ANF release was examined in atria from rats pretreated subcutaneously with 5 mg/kg of reserpine (Serpasil ampule, CIBA Pharmaceutical, Summit, New Jersey) for 2 days. This treatment reduces cardiac catecholamine content to undetectable levels, and isolated atria were refractory to challenges with 10⁻³ M tyramine. Chronotropic stimulation was carried out as described above.

To probe whether physicochemical membrane phenomena such as sodium conductance were involved in chronotropic-mediated ANF, IRANF secretion was determined in paced atria incubated with lidocaine (lidocaine HCl, ICN Immunobiologicals, Planview, New York). A concentration of 5x10⁻⁴ M was used because this has been shown to block sodium conductance in isolated cardiac preparations.

Statistics

Although values of IRANF are presented as the arithmetic mean±SEM, data were statistically evaluated in the log scale as required for data generated from a RIA log concentration standard curve. Responses were characterized by slope of log ANF versus atrial rate and compared on the basis of log-fold increase above baseline IRANF release. Slopes were analyzed by analysis of variance to determine differences among groups. Correlations of ANF release with systolic tension were determined by least-squares method of linear regression.

Results

Effect of Resting Tension on ANF Release

For the analysis of heart rate effects on ANF secretion, it was first necessary to determine the
amount of IRANF released by constant resting tension in nonstimulated atria (Table 1). These values were determined immediately prior to electrical stimulation and subtracted from the IRANF released in response to pacing. It was noted that despite a steady resting tension, the quantity of IRANF release declined by as much as 50% during the second 10-minute collection period compared with the initial 10-minute period. The reduction in secretion could not be attributed to degradation of ANF, since recovery of synthetic ANF added to the bath was essentially 100%.

Effect of Electrical Stimulation (1–5 Hz) at Constant Resting Tension

As presented in Figure 1, IRANF secretion was unaffected by electrical pacing (1–5 Hz) when atria were maintained at 1 g of resting tension. In contrast, electrical pacing elevated IRANF secretion in a frequency-dependent manner in atria held constant at higher resting tensions of 2, 3, and 4 g. As was observed with unpaced atria (Table 1), IRANF release was maximal during the first 10-minute period of stimulation and decreased during the next 10 minutes despite continuous pacing. This was evident at all stimulation frequencies.

The magnitude of the IRANF secretory response to chronotropic stimulation was dependent on the degree of resting tension. In atria paced at 2 or 5 Hz, a diastolic tension of 3 g appeared to be the maximally effective resting tension for rate-stimulated ANF release since electrical stimulation at 4 g released threefold less IRANF than at 3 g. When compared on the basis of log-fold increase over baseline (statistical analysis not shown), significantly (p<0.05) more IRANF was released with atrial pacing (2 Hz) at 2 and 3 g of resting tension than at 1 or 4 g.

Effect of High-Frequency Stimulation (8 or 9 Hz) on ANF Release at Constant Resting Tension

The effect of electrical stimulation at rates of 8 Hz on ANF release was investigated in atria at 0.5, 1, and 4 g of resting tension. These tensions were used because they were insensitive to rate-induced ANF release with pacing up to 6 Hz (e.g., 0.5 and 1 g) or exhibited reduced responsiveness to ANF release induced by electrical pacing (e.g., 4 g). At these resting tensions, high-frequency stimulation was effective in increasing ANF release (Figure 2). The quantity of IRANF secreted in response to 8 Hz at 1 g of tension was comparable to the amount released at lower frequencies but at higher resting tensions (2 or 3 g, Figure 1). No additional enhancement of IRANF secretion was observed with 9 Hz in atria maintained at a diastolic tension of 1 g (IRANF, 6.5±1.2 ng/10-min period; 10.9±2.2 ng/20 min). As noted with lower frequencies, ANF release decreased over the 20-minute collection period despite continuous high-frequency pacing.

Molecular Weight Estimate of Released ANF

As assessed by molecular weight size separation and fraction analysis, the low molecular weight form of ANF (3,000 Da) was released with resting tension and pacing manipulations. The size of ANF

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**Table 1.** IRANF Release at Different Resting or Diastolic Tensions in Unpaced Rat Left Atria

<table>
<thead>
<tr>
<th>Resting tension*(g)</th>
<th>IRANF released (ng/atria/10 min)</th>
<th>IRANF released (ng/atria/20 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.58±0.70</td>
<td>10.3±0.86</td>
</tr>
<tr>
<td>2</td>
<td>5.35±0.66</td>
<td>7.3±0.90</td>
</tr>
<tr>
<td>3</td>
<td>8.27±0.73</td>
<td>11.2±1.43</td>
</tr>
<tr>
<td>4</td>
<td>9.68±0.92†</td>
<td>14.07±0.96†</td>
</tr>
</tbody>
</table>

* A fixed diastolic tension was applied and immunoreactive atrial natriuretic factor (IRANF) was sampled from the bathing solution at 10 and 20 minutes. For purposes of this study, the values in this table were defined as baseline IRANF release, independent of atrial rate, and were subtracted from IRANF released during pacing protocols. Each value is the mean±SEM of six atria.

† Immediately before electrical stimulation a diastolic tension of 1–4 g was imposed on the isolated left atria.

† Significantly (p<0.05) different from release at 1 g.
released in vitro approximated that of the in vivo circulating physiologically active form and was clearly distinct from the stored or unprocessed ANF (13,000 Da). Furthermore, LDH release, 0.06% of the total LDH atrium content, was not changed by experimental conditions (0 resting tension to 4 g, 8 Hz), indicating irreversible damage of cells cannot account for release of IRANF in our preparations.

Correlation Between Rate-Induced IRANF Release and Developed Tension

The rat, unlike most other mammals, exhibits a negative force-frequency relation in which an increase in contraction frequency is associated with a decrease in developed tension.14 In the present experiments, enhanced IRANF release due to increased contraction frequency was accompanied with a reduction in developed or systolic tension. Because the relation between IRANF release and systolic tension involves a third variable, pacing frequency, a single correlation between systolic tension and IRANF secretion, although negatively associated in time, was not statistically possible. Therefore, statistical evaluation of the relation between IRANF release and systolic tension was carried out within each of two stimulation frequencies and two resting tensions. The results of this statistical approach are presented in Figure 3 and show that within defined conditions, IRANF secretion was positively correlated with developed tension. At each frequency, the slope of the regression line was influenced by the resting tension such that greater slopes were observed with 3 versus 2 g resting tension. As expected from the above results, more IRANF was released at 5 versus 2 Hz stimulation frequency and at 3 versus 2 g of resting tension. Therefore, the results confirm that resting tension is a determinant of IRANF release and further show rate and developed tension also contribute to release of this peptide.

Effect of Reserpine Pretreatment on Chronotropic-Stimulated IRANF Release

Baseline release in unpaced atria stretched to 2 or 3 g of diastolic tension was not significantly influenced by catecholamine depletion (Table 2). Frequency-induced IRANF release at 2 and 3 g of tension was reduced in atria removed from reserpinized rats compared with control rats; the extent of depressed rate-induced IRANF release was greater at the highest degree of stretch (3 g tension; Table 2). It is pointed out that release data in this experiment was obtained from atria of control and reserpinized animals stimulated at different but overlapping frequencies. We, therefore, chose to make group comparison with a slope analysis (fold increase) of IRANF release per 60 beats per minute change in contraction frequency.

Effect of Lidocaine on Rate-Induced Release

Total IRANF release from stimulated atria (6 Hz) at resting tension of 1, 2, and 3 g was not significantly altered by the presence of a single concentration (5×10⁻⁴ M) of lidocaine. (IRANF, ng/atrium/10 min:...
Atrial contraction frequency has been proposed to be a stimulus for release of ANF, and data showing an increase in plasma or perfusate ANF following atrial pacing in vivo and in vitro support this proposal. To date the question as to whether contraction frequency is a primary stimulus for ANF secretion has not been answered. It is possible that contraction frequency releases ANF merely as a consequence of changes in atrial stretch, since changes in atrial pressure or tension accompany paroxysmal tachycardia, intracardiac pacing, or electrical stimulation of isolated atria. Our results clarify this issue by demonstrating that chronotropic stimulation effectively releases ANF in the absence of a concurrent elevation in diastolic tension (degree of stretch) and in the absence of an increase in systolic tension.

Release of ANF with chronotropic stimulation exhibited several interesting characteristics. It was found that the ANF secretory response induced by a change in atrial rate was influenced by both the pacing frequency and by the preestablished level of diastolic tension. Specifically, at low resting tensions (1 g or less), atrial rates (60–300 beats/min) up to slightly less than the normal heart rate for the rat were ineffective in promoting ANF release. In contrast, atria stretched to 2 g or more of resting tension and stimulated to beat at these same rates (60–300 beats/min) release ANF in a rate-dependent manner in amounts exceeding that promoted by a change in stretch alone. Higher pacing frequencies (atrial rate >300 beats/min) elevated ANF release regardless of the predetermined resting tension. Furthermore, as judged by molecular weight sizing and LDH release, abnormal release of the large molecular weight stored ANF or myocyte damage could not account for the secretory responses observed here. Thus, it is evident from our results that from 60–540 beats/min, the magnitude of the secretory response of the low molecular weight, physiologically active ANF peptide was influenced by the frequency and the degree of steady-state resting tension.

From our present findings and those of others, atrial ANF secretion in vitro appears to occur in response to at least three separate but possibly related stimuli or conditions: 1) basal or spontaneous, independent of stretch or rate; 2) stretch-induced, independent of rate; and 3) rate-induced, at elevated diastolic tension and/or rapid rates. It is speculated that this latter phenomenon of rate-induced ANF release, the focus of our study, provides a possible explanation for the extreme elevation in plasma ANF concentration evident in congestive heart failure and clinical tachycardias. The dual existence of both elevated atrial distension and heart rate in these clinical conditions would not only promote ANF release by two separate stimuli but also serve to potentiate the magnitude of rate-induced ANF secretory response, a situation analogous to the frequency-release observed at 3 g in this study. In particular, this interpretation seems applicable to a recent report in which the concentration of plasma ANF of patients with ventricular tachycardia was approximately threefold higher than those with supraventricular tachycardia, although both were elevated above control. The coexistence of high atrial pressures and elevated atrial rates in supraventricular tachycardia, compared with the lower atrial pressures and increased rates in supraventricular tachycardia, may account for the difference between plasma ANF levels in these two clinical conditions.

To develop a statistical relation between systolic tension and ANF release, it was necessary to relate systolic tension and ANF secretion at a single diastolic tension and single frequency of stimulation. At each set of pacing frequency and diastolic tension, a positive correlation between systolic tension and ANF secretion was noted. The physiological significance of this correlation is limited, since...
the largest quantities of ANF were released at rapid atrial rates which, in the rat, are associated with decreased systolic tension. Although systolic tension is important in promoting ANF release, we have interpreted these results to mean that, at least in the rat and hamster, species which exhibit a negative force-frequency relation,14 systolic tension exerts a minor influence on ANF secretion at rapid heart rates.

It was consistently observed that ANF secretion during continuous chronotropic stimulation, and with chronic application of constant resting tension, decreased with time. These findings confirm a recent publication demonstrating the transient nature of ANF release with stretch.28 Our results extend these observations by showing that, in addition to stretch, rate-induced ANF secretion also declines with time. It is possible that the time course of change of ANF secretion in vitro may be modified by additional factors in vivo, as suggested by the observation that ANF release following electrical pacing in the open-chest rabbit increased gradually over 20 minutes.29 At present, this issue is unresolved since difference in species and sampling protocols may explain the various time courses.29

Several reports suggest that the cellular mechanism for ANF release may be atypical.30,31 As judged by electronmicrographic analysis of atrial tissue, an exocytic pathway of vesicle plasma membrane fusion appears to be a rare event for ANF-containing granules.30 In addition, extracellular calcium is apparently not required for cellular release of ANF by osmotic stimuli.31 Although involvement of the phosphoinositol pathway in ANF release has been proposed,32,33 concurrent changes in pressure and rate produced by phorbol esters and calcium ionophores have complicated interpretation of data on which this proposal is based. Our studies have focused on catecholamines and sodium conductance as possible molecular mediators of release by chronotropic stimulation. Since catecholamines have also been shown to stimulate ANF secretion from atrial tissue34 and atrial myocytes15 and since catecholamines may also be released under conditions of electrical stimulation in isolated atrial preparations, it seemed possible that catecholamines could influence ANF release of atrial contraction. Results of a previous publication,13 in which ANF release was measured after α- and β-blockade, suggested that catecholamines were not involved in this type of release. Our results show that at 2 and 3 g of resting tension, higher tensions than used previously,13 a reduced ANF secretory response with pacing was observed in reserpинized atria, suggesting that catecholamines may mediate frequency-stimulated ANF release under certain conditions. Although the effect of reserpine on the biosynthesis, storage, and release of ANF is unknown, a possible direct depressant effect of reserpine on cellular secretion cannot be excluded. In light of data showing an increase in ANF storage with a prolonged decrease in atrial pressure,15 it seems more likely that reserpine-induced hypotension of 48-hour duration would serve to increase ANF storage pools and so facilitate, not depress, rate-induced release. Our tentative conclusion is that in the distended atria (3 g), catecholamine release contributes to ANF release stimulated by pacing. However, a direct effect of reserpine needs further investigation.

A second approach to investigating the mechanism of rate-induced ANF release, was to evaluate release after disturbing the physicochemical aspects of the membrane with a local anesthetic, lidocaine. It was reasoned that since stretch and rate, effective stimuli for ANF release, both induce mechanical distortions of the membrane and since rate additionally influences membrane conductance, the interference in these phenomena would block ANF release. At a dose previously shown to reduce sodium conductance in cardiac tissue,18,19 lidocaine was ineffective in altering ANF secretion following pacing. These results suggest that sodium conductance through channel receptors sensitive to a local anesthetic is not involved in release of ANF with pacing. In these initial experiments, no attempt was made to sort out differential effects of lidocaine on release occurring basally by stretch or rate.

In summary, our findings on chronotropic stimulation of ANF release indicate that atrial rate is a determinant of ANF secretion, separate from atrial stretch. The magnitude of this phenomenon depends not only on stimulation frequency but, interestingly, on the level of preestablished diastolic tension. The molecular mechanism for rate-related release has not been established in this study, although a major role for catecholamine or sodium conductance was not evident.

Acknowledgments

The authors express their appreciation to Mr. George Morgan and Mr. Carl Homnick for the expert technical assistance and to Mrs. Joan Brooke for the secretarial preparation of the manuscript.

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Key Words • atrial natriuretic factor • atrial rate • diastolic tension • secretion • rat
Chronotropic stimulation: a primary effector for release of atrial natriuretic factor.
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Circ Res. 1989;64:799-805
doi: 10.1161/01.RES.64.4.799

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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