Neuropeptide Y as a Putative Modulator of the Vagal Effects on Heart Rate

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Neuropeptide Y is stored in sympathetic nerve terminals throughout the heart and has direct and indirect effects on cardiac function. Although neuropeptide Y has been shown to be released upon intense (16–30 Hz) cardiac sympathetic stimulation, we sought to determine whether effective quantities of neuropeptide Y were released from cardiac sympathetic neurons under more natural conditions. We recorded arterial pressure and cardiac cycle length in 29 anesthetized dogs. We assessed neuropeptide Y release by measuring the attenuation of vagally induced increases in cardiac cycle length (10 seconds every 2 minutes) after trains of sympathetic stimulation. We examined the effect of constant-frequency sympathetic stimulation (frequencies of 2, 5, 10, and 15 Hz, applied for train durations of 1, 3, and 5 minutes) on vagally induced chronotropic responses. We also determined the effect of varying the pattern of sympathetic stimulation. Both the magnitude and duration of the inhibition of the vagal effects on cardiac cycle length were augmented significantly by increases in the frequency or duration of sympathetic stimulation. In contrast, the inhibition of the vagally induced chronotropic responses was not significantly affected by changes in the pattern of sympathetic stimulation. We also characterized the role of adrenergic receptors. Phentolamine significantly increased the sympathetically mediated inhibition of the vagal effects on cardiac cycle length, but propranolol had no effect. We concluded that neuropeptide Y release from cardiac sympathetic neurons 1) depends on the frequency and duration, but not on the pattern, of sympathetic stimulation; 2) is evoked even at physiological frequencies (2–5 Hz) of sympathetic activity; and 3) is enhanced by α-adrenergic-receptor blockade, but is unaffected by β-adrenergic-receptor blockade. (Circulation Research 1989;64:882–889)
Neuropeptide Y attenuated the negative chronotropic and inotropic responses evoked by field stimulation. Furthermore, in this preparation exogenous neuropeptide Y did not significantly modify the inhibitory responses elicited by exogenous acetylcholine. Similarly, Potter showed in the anesthetized dog that increases in cardiac cycle length evoked by bethanechol were not affected by exogenous neuropeptide Y or by sympathetic stimulation, whereas vagally induced increases in cycle length were inhibited by either sympathetic stimulation or exogenous neuropeptide Y. Hence, the inhibitory effect of sympathetic stimulation (presumably mediated by neuropeptide Y) appears to occur prejunctionally, on the vagal nerve endings, rather than postjunctionally, on the cardiac cell membrane.

Although norepinephrine and neuropeptide Y are released concomitantly upon sympathetic activation, substantial evidence suggests that much higher stimulation frequencies are required to release detectable quantities of neuropeptide Y than of norepinephrine. The pattern of stimulation also substantially affects the release of neuropeptide Y from splenic nerve fibers in the pig and calf. The location of neuropeptide Y in sympathetic nerve endings throughout the heart suggests that neuropeptide Y can modulate all aspects of cardiac function. Therefore, we sought to determine in more detail the stimulation conditions that release neuropeptide Y from cardiac sympathetic neurons. In our experiments we monitored the sympathetically mediated inhibition of the vagal effects on cycle length as an indirect measure of vagally induced chronotropic responses. We also determined the role of \( \alpha \) and \( \beta \)-adrenergic receptors in the sympathetically mediated inhibition of vagally induced chronotropic responses.

### Materials and Methods

#### Preparation

Experiments were carried out on 29 mongrel dogs (14–27 kg) that were premedicated with morphine sulfate (2 mg/kg i.m.) and anesthetized with \( \alpha \)-chloralose (75 mg/kg i.v.). A femoral artery was cannulated for arterial pressure measurement (Statham model P23AC, Gould, Cleveland, Ohio). The ipsilateral femoral vein was cannulated for the administration of drugs and the maintenance of fluid balance. The cervical vagi were isolated, doubly ligated, and sectioned to interrupt descending parasympathetic outflow to the heart. Bipolar hook electrodes were inserted into the cardiac end of each vagus, and the wires were connected in parallel to a stimulator (Grass Instrument, Quincy, Massachusetts).

The trachea was intubated and intermittent positive pressure ventilation was begun. The chest was opened transversely at the fourth intercostal space. The right and left stellate ganglia were isolated, doubly ligated, and sectioned to eliminate descending cardiac sympathetic outflow. The ansae subclaviae were placed over bipolar shielded iridium electrodes (Harvard Apparatus, South Natick, Massachusetts), and the electrode wires were connected in parallel to a Grass stimulator. The pericardium was opened, and a bipolar electrode catheter was inserted into the right atrial appendage to record an atrial electrogram. Arterial blood pressure, right atrial electrogram, and A-A interval were recorded continuously (model ES 1000, Gould). The A-A interval (cardiac cycle length) was determined from the atrial electrogram by an analog computer (model 580, Electronic Associates, Inc, West Long Beach, New Jersey).

#### Stimulation Conditions

The vagi were stimulated supramaximally (6–8 V, 1-msec pulse width) for 10 seconds every 2 minutes. The vagal stimulation frequency was adjusted to elicit approximately a 100% increase in cardiac cycle length under control conditions. The vagal stimulation frequencies ranged from 1.5 to 6 Hz in individual animals.

Two modes of supramaximal stimulation (8 V, 1-msec pulse width), either constant or patterned stimulation, were applied to the ansae subclaviae. The constant stimulation was delivered to the ansae subclaviae at frequencies of 2, 5, 10, and 15 Hz for train durations of 1, 3, and 5 minutes. The order of application of the combinations of frequency and duration was randomized according to a Latin-square design. Specific protocols involving constant frequency stimulation are described in the results.

For the second stimulation mode, patterned stimulation, we programmed a microprocessor (model ET-3400A, Heath Co, Benton Harbor, Michigan) to generate continuous (100% duty cycle) and intermittent (50% or 25% duty cycle) sympathetic stimulation patterns. The stimulation patterns were delivered to the sympathetic nerves at mean frequencies of 2, 5, and 10 Hz, each for a train duration of 3 minutes. The protocol used to deliver continuous and intermittent stimulation patterns at a mean frequency of 5 Hz with a 1-second subperiod is shown in Figure 1. During continuous stimulation, five pulses were delivered over 100% of each 1-second subperiod. For a 50% duty cycle, the five pulses were delivered during half of each 1-second subperiod, and no pulses were delivered during the remaining half of the subperiod. Similarly, for a 25% duty cycle, the five pulses were delivered in 0.25 second, and no pulses were delivered in the remaining 0.75 second of the subperiod.

We also programmed the microprocessor to generate patterned sympathetic stimulation delivered at mean frequencies of 2, 5, and 10 Hz, but based on
Figure 1. Protocol used to deliver different patterns of stimulation to ansae subclaviae at a mean frequency of 5 Hz. During continuous stimulation, five pulses were delivered over 100% of each 1-second subperiod. The 100% duty cycle is analogous to constant frequency stimulation at 5 Hz. Intermittent patterns were applied at a 50% and a 25% duty cycle. Note that although patterns of stimulation differ, total numbers of pulses delivered each second is constant.

Figure 2. Vagally induced increases in cardiac cycle length in a representative experiment before and after supramaximal stimulation of the ansae subclaviae (10 Hz for 5 minutes). The vagi were stimulated supramaximally for 10 seconds every 2 minutes, except during sympathetic stimulation. Although the identical vagal stimulation was applied before and after sympathetic stimulation, the vagally induced increases in cardiac cycle length after sympathetic stimulation were attenuated for 36 minutes.
Figure 3 shows a representative example of the recovery of vagally induced cardiac cycle-length responses (expressed as percent of control) after the termination of 5-minute trains of sympathetic stimulation delivered at frequencies of 5, 10, and 15 Hz. Note that as the frequency of sympathetic stimulation was incremented from 5 to 15 Hz, the initial inhibition (at \( t=2 \) minutes) of the vagal effects on cycle length increased and the time course of recovery was more prolonged. For example, after sympathetic stimulation at 5 Hz, the initial chronotropic response to vagal stimulation was inhibited by 53%, whereas after sympathetic stimulation at 15 Hz, the initial chronotropic response to vagal stimulation was inhibited by 86%. The corresponding times for the chronotropic responses to return to their control values were about 20 and 60 minutes, respectively.

**Composite Data**

**Effect of level of sympathetic stimulation.** We determined the effect of the frequency and duration of sympathetic stimulation on the vagally induced chronotropic responses by stimulating the ansae subclaviae at 5, 10, and 15 Hz and applying each frequency for train durations of 1, 3, and 5 minutes. For each of the nine sympathetic stimulation combinations we quantitated the initial inhibition, which we defined as the first vagally induced chronotropic response (i.e., at \( t=2 \) minutes in Figure 3) after the termination of sympathetic stimulation. The initial inhibition was calculated as \( 100(R_c-R_e)/R_c \), where \( R_c \) is the control vagally induced cycle-length response (before sympathetic stimulation) and \( R_e \) is the first experimental response (after sympathetic stimulation). The initial inhibition data from five animals are summarized in the top panel of Figure 4. Analysis of variance showed that changes in the frequency \(( p=0.005)\) and duration \(( p=0.002)\) of the sympathetic stimulation train significantly affected the initial inhibition of the chronotropic response to vagal stimulation.

We also quantitated the summated inhibition, which we defined as the area between the response curve and the horizontal line that represents 100% of the control response (e.g., as in Figure 3). This area represents the effect of the train of sympathetic stimulation on the chronotropic responses to vagal stimulation summated over the full duration of the response. The summated inhibition data are summarized in the bottom panel of Figure 4. Analysis of variance revealed that changes in the frequency \(( p=0.01)\) and duration \(( p=0.02)\) of sympathetic stimulation significantly affected the summated inhibition.

We determined the relation between the initial and summated inhibition of the vagal effects on cardiac cycle length by plotting each summated inhibition versus its corresponding initial inhibition. Figure 5 shows a plot of these data, which were represented by an equation that we determined by nonlinear regression analysis as

\[
Y = 0.21X^2
\]

![Figure 4](http://circres.ahajournals.org/)

**Figure 4.** Effects of frequency and duration of sympathetic stimulation on initial (top panel) and summated (bottom panel) inhibition of vagally induced chronotropic responses. Initial and summated inhibitions are plotted versus durations of sympathetic stimulation, in minutes. Each of the frequencies of sympathetic stimulation is represented by a curve labeled 5, 10, or 15 Hz. Data points represent mean±SEM (n=5).
FIGURE 5. Relation between summated inhibition and initial inhibition of vagal effects on cardiac cycle length evoked by sympathetic stimulation. Data points represent initial and summated inhibition obtained after 5-, 10-, and 15-Hz sympathetic stimulation delivered using train durations of 1, 3, and 5 minutes in five animals. Data shown in Figure 4 represent mean±SEM of these data.

The square of the curvilinear correlation coefficient ($R^2$) for the data shown in Figure 5 is 0.87. Thus, 87% of the relation between the initial and summated inhibition is accounted for by the above regression equation; $R^2$ serves as a criterion of the goodness of fit.

Effect of pattern of sympathetic stimulation. We followed the stimulation protocol used in the experiment illustrated in Figure 2 to compare the effects of continuous and intermittent sympathetic stimulation patterns on the vagally induced chronotropic responses. We initially tested the effects of three different duty cycles (25%, 50%, and 100%) and three different frequencies (2, 5, and 10 Hz) of sympathetic stimulation, each delivered for a train duration of 3 minutes. The various combinations of duty cycles and stimulation frequency were applied in a random sequence. For each 3-minute train of sympathetic stimulation, we calculated the initial and summed inhibition of the vagally induced chronotropic responses. The data ($n=5$) are summarized in Figure 6. The mean frequency of sympathetic stimulation significantly affected the initial inhibition ($p<0.001$, top panel) and summed inhibition ($p<0.001$, bottom panel). Thus, as the mean frequency of sympathetic stimulation was incremented, both the initial inhibition and the summed inhibition were significantly augmented. In contrast, at any given mean frequency, the pattern of sympathetic stimulation (i.e., 25%, 50%, or 100% duty cycle) did not significantly affect the initial inhibition ($p=0.9$) or the summed inhibition ($p=0.9$).

To determine whether various stimulation patterns might evoke different effects if longer subperiods were used, we next examined sympathetic stimulation patterns delivered with a 10-second subperiod. During each 10-second subperiod we applied a 20-Hz train of stimulation for 1, 2.5, or 5 seconds, but delivered no pulses during the remaining portion of the subperiod; these patterns corresponded to mean frequencies of 2, 5, and 10 Hz. We compared the effect of the intermittent stimulation with the effect of continuous sympathetic stimulation delivered at frequencies of 2, 5, and 10 Hz. The initial and summed inhibition data are summarized in Figure 7 ($n=5$). The mean frequency of sympathetic stimulation significantly affected the initial inhibition ($p=0.001$, top panel) and summed inhibition ($p=0.001$, bottom panel). At any given mean frequency (i.e., 2, 5, or 10 Hz), however, the initial inhibition after the termination of continuous (bars labeled C) stimulation was not significantly different ($p=0.6$) from the initial inhibition after the termination of intermittent (bars labeled I) sympathetic stimulation. Similarly, at any given mean frequency, the summed inhibition was not significantly ($p=0.8$) affected by the pattern (continuous versus intermittent) of sympathetic stimulation. The data in Figures 6 and 7 show that the mean frequency of sympathetic stimulation significantly influences the inhibition of the vagal effects on cardiac cycle length but that the pattern of sympathetic stimulation does not, regardless of the frequency of stimulation.
Role of adrenergic-receptor blockade. The release of norepinephrine from sympathetic nerve terminals is altered by \(\alpha\)- and \(\beta\)-adrenergic-receptor blockade.\(^{12,14}\) Because neuropeptide Y is also released from sympathetic nerve terminals,\(^{11,13,18}\) we sought to determine whether \(\alpha\)- or \(\beta\)-adrenergic-receptor antagonists modulated the release of neuropeptide Y. In addition, we examined whether the norepinephrine-induced decrease in cardiac cycle length during sympathetic stimulation (e.g., Figure 2) influenced the persistent inhibition of the vagal effects on cardiac cycle length.

Animals were assigned randomly to a control (\(n=5\)), propranolol (\(n=4\)), or phentolamine (\(n=5\)) group. For each group the experiments were subdivided into two observation periods. During the first observation period, six trains of stimulation (2 and 5 Hz, each for train durations of 1, 3, and 5 minutes) were delivered in random sequence to the ansae subclaviae. We followed the protocol used in the experiment shown in Figure 2 to determine the effects of each combination of sympathetic stimulation frequency and train duration on the chronotropic responses to vagal stimulation. At the end of observation period 1, we administered saline, propranolol, or phentolamine to the animals in the control, propranolol, or phentolamine groups, respectively. During observation period 2, we repeated the six trains of sympathetic stimulation after a new randomization. In the control group, comparison of the responses obtained during periods 1 and 2 provided information about the changes in cardiac responses over time. In the propranolol and phentolamine groups, period 1 served as an internal control for the effects of propranolol and phentolamine, respectively.

The initial inhibition data are summarized in Figure 8. In the control group and in the group that received propranolol (Figures 8A and 8B and Figures 8C and 8D, respectively), the initial inhibitions obtained during period 1 were not significantly different (\(p=0.7\) and \(p=0.3\), respectively) from the initial inhibitions obtained during period 2. In the group that received phentolamine (Figures 8E and 8F), however, the initial inhibitions were significantly augmented (\(p<0.001\)) after phentolamine (period 2) at sympathetic stimulation frequencies of either 2 or 5 Hz.

Qualitatively similar results were obtained for the summed inhibition in each of the three experiment-
tial groups (data not shown). In the control and propranolol groups, the summed inhibitions in period 1 were not significantly different from the summed inhibitions in period 2 (p = 0.9 and p = 0.6, respectively). In the phentolamine group, however, the summed inhibitions were significantly augmented after phentolamine (p < 0.001). Therefore, \(\alpha\)-adrenergic-receptor blockade significantly increased the magnitude and duration of the sympathetically induced inhibition of the vagal effects on cardiac cycle length, whereas neither \(\beta\)-adrenergic-receptor blockade nor the passage of time affected the inhibition appreciably.

Discussion

Our results showed that the inhibition of the vagal effects on cardiac cycle length that is evoked by sympathetic stimulation 1) depends on the frequency and duration of sympathetic stimulation, 2) occurs even at physiological frequencies (2–5 Hz) of sympathetic stimulation, 3) is not significantly affected by substantial changes in the pattern of sympathetic stimulation, 4) is significantly enhanced by \(\alpha\)-adrenergic-receptor blockade, and 5) is unaffected by \(\beta\)-adrenergic-receptor blockade. The long-lasting inhibition of the vagally induced chronotropic responses that occurs after sympathetic stimulation is probably ascribable to the release of neuropeptide Y from the sympathetic nerve endings. Because neuropeptide Y antagonists are not available to date, however, we cannot rule out the possibility that another mechanism may be at least partly responsible for the observed results.

In the present experiments we used a protocol similar to that described by Potter\(^{15}\) to indirectly measure the release of neuropeptide Y from cardiac sympathetic neurons in the anesthetized dog. Potter showed that the vagal actions on cardiac cycle length were attenuated after short periods of intense sympathetic stimulation (i.e., at frequencies of 16–20 Hz). Our experiments confirm Potter's results, because we also found that high-frequency sympathetic stimulation (e.g., 10 and 15 Hz) persistently inhibited the vagal effects on cardiac cycle length (Figures 2–4). Moreover, our data extend Potter's previous studies by demonstrating that 1) both the magnitude (initial inhibition) and duration (summed inhibition) of the inhibition of vagally induced chronotropic responses are significantly affected by the frequency and duration of sympathetic stimulation (Figures 3 and 4), and 2) even low frequencies of sympathetic stimulation (2–5 Hz) can inhibit the vagal effects on cardiac cycle length (Figures 3, 4, 6–8). This latter observation is important because functional effects of neuropeptide Y have not previously been reported in response to low levels of sympathetic stimulation. The release of neuropeptide Y at naturally occurring frequencies of sympathetic activity (e.g., 5 Hz) suggests that neuropeptide Y may act to regulate cardiac function under physiological conditions. In support of this conclusion, Potter\(^{15}\) demonstrated that cardiac vagal effectiveness was inhibited after a reflexly mediated increase of sympathetic activity.

Lundberg et al\(^ {13}\) showed that in the isolated pig spleen, the neuropeptide Y release was much greater when the splenic nerve was stimulated with short bursts of 20-Hz stimulation than when the nerve was stimulated with the same number of pulses, but at a steady low frequency (2 Hz) of stimulation. Similarly, Allen et al\(^ {18}\) demonstrated in the conscious calf that the mean plasma neuropeptide Y concentration was significantly greater after stimulation of the splenic nerves with 40-Hz bursts of stimuli than when the same number of stimuli was delivered with continuous low-frequency (4-Hz) stimulation. These studies showed that the pattern of stimulation influenced the release of neuropeptide Y from the sympathetic neurons of the spleen of pigs and calves. In our experiments, changing the pattern of cardiac sympathetic stimulation did not significantly alter the chronotropic response to vagal stimulation in dogs (Figures 6 and 7). The inhibition of the vagal effects on cardiac cycle length, however, was significantly affected by the frequency and duration of sympathetic stimulation (Figures 3, 4, 6–8). Thus, in the dog heart neuropeptide Y release is affected by the total number of impulses delivered to the sympathetic nerves, but is not very sensitive to the pattern with which those impulses are delivered, at least with the varieties of patterns that we examined in our study.

Several factors may account for the differences between our observations in the dog heart and those obtained in the spleens of pigs and calves. Lundberg et al\(^ {13}\) and Allen et al\(^ {18}\) measured directly the amount of neuropeptide Y released during splenic nerve stimulation. However, we indirectly measured neuropeptide Y release by monitoring a functional response (i.e., the neuropeptide Y-induced inhibition of vagally induced chronotropic responses). Thus, it is possible that in our preparation the quantity of neuropeptide Y release did change in response to the different patterns of cardiac sympathetic stimulation, but that the change in neuropeptide Y release did not appreciably affect the functional response. This possibility is unlikely, however, because our data demonstrate that the inhibition of the cardiac vagal effects was intensified significantly as we increased the frequency or duration of sympathetic stimulation (Figures 3, 4, 6–8). Therefore, if the different sympathetic stimulation patterns used in our study produced significant changes in neuropeptide Y release, those changes must have been substantially less than those evoked by the changes we induced in the duration or mean frequency of sympathetic stimulation. Alternatively, the differences in the effect of pattern of sympathetic stimulation on neuropeptide Y release in our studies and in the experiments of Lundberg et al\(^ {13}\) or Allen et al\(^ {18}\) may reflect a tissue or species variation.
Activation of the α-adrenergic receptors located on sympathetic nerve terminals inhibits the release of norepinephrine from those nerve endings, whereas blockade of α-adrenergic receptors enhances norepinephrine release.\textsuperscript{12,14} Data obtained in the pithed guinea pig and in the anesthetized pig show that the stimulation-evoked release of neuropeptide Y from sympathetic nerve endings is also enhanced after the blockade of α-adrenergic receptors.\textsuperscript{11,20} Indeed, Rudehill et al\textsuperscript{11} showed that the sympathetic stimulation-evoked overflow of neuropeptide Y from the heart increased threefold after α-adrenergic-receptor blockade. Our data extended these findings by demonstrating that the inhibition of cardiac vagal effects resulting from a given frequency of sympathetic stimulation was significantly greater after α-adrenergic-receptor blockade than under control conditions (Figure 8). Thus, in the heart, the effect of α-adrenergic-receptor blockade on neuropeptide Y release is functionally significant. Additionally, our results with adrenergic receptor antagonists extended the data of Potter.\textsuperscript{19} Although Potter demonstrated that the sympathetically mediated inhibition of the vagal actions on heart rate remained after combined α- and β-adrenergic-receptor blockade, she did not analyze separately the effects of α- and β-adrenergic-receptor antagonists. Our data showed that the inhibition of cardiac vagal effects evoked by sympathetic stimulation is unaffected by β-adrenergic-receptor blockade, but is significantly enhanced by α-adrenergic-receptor blockade (Figure 8). Although the inhibition of the vagal effects on cycle length is nonadrenergic in origin, the inhibition is modulated by α-adrenergic-receptor blockade.

Note added in proof: After we submitted this manuscript, a relevant study by T.D. Gardner and E.K. Potter was published in the Journal of Physiology (Dependence of non-adrenergic inhibition of cardiac vagal action on peak frequency of sympathetic stimulation in the dog. J Physiol [Lond] 1988;405:115–122). In contrast to our results (Figures 6 and 7), which show that the pattern of sympathetic stimulation did not affect the inhibition of vagally induced cycle length responses, Gardner and Potter show that the pattern of stimulation did affect the inhibition of vagal effects on cycle length. The reasons for this discrepancy are unclear.

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