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)

The autonomic nervous system modulates cardiac chronotropic, inotropic, and dromotropic processes.1,2 Our current understanding of the autonomic control of the heart is based mainly on the effects of norepinephrine and acetylcholine, neurotransmitters that are released from the cardiac sympathetic and parasympathetic nerves, respectively.1,2 Recently, however, it has been shown that autonomic neurons, including those to the heart, contain various neuropeptides in addition to the "classical" neurotransmitters.3–7 These neuropeptides may act as neurotransmitters or neuromodulators.

The most abundant neuropeptide found in cardiac autonomic nerves is neuropeptide Y, a 36-amino acid peptide that coexists with norepinephrine in cardiac sympathetic nerve terminals.3,4,6–10 Neuropeptide Y–immunoreactive nerves are located in the atrial and ventricular myocardium and near the coronary arteries.3,4,7,8 Dense populations of such nerves are also present in the sinoatrial and atrioventricular nodal regions.4,7,8

Neuropeptide Y is released together with norepinephrine upon direct stimulation of sympathetic fibers.11–14 In the anesthetized dog, Potter15 demonstrated that the vagal actions on heart rate were attenuated after brief periods of intense sympathetic stimulation. Potter attributed the inhibition of the vagal effects on heart rate to the release of neuropeptide Y from sympathetic nerve endings, because 1) the inhibition remained in the presence of adrenergic-receptor blockade, and 2) the inhibition was mimicked by exogenous neuropeptide Y.15,16 Additional evidence suggests that neuropeptide Y attenuates cardiac vagal actions by inhibiting the release of acetylcholine from postganglionic parasympathetic neurons.16,17 Lundberg et al17 showed that in the isolated guinea pig atrium, after β-adrenergic-receptor blockade exogenous neu-
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\textsuperscript{16} 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 coherently upon sympathetic activation,\textsuperscript{11-14,19} substantial evidence suggests that much higher stimulation frequencies are required to release detectable quantities of neuropeptide Y than of norepinephrine.\textsuperscript{13,15,18} The pattern of stimulation also substantially affects the release of neuropeptide Y from splenic nerve fibers in the pig\textsuperscript{13} and calf.\textsuperscript{18} 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 neuropeptide Y release. We examined the effects of the level (i.e., frequency and duration) of sympathetic stimulation as well as the effect of the pattern of sympathetic stimulation on the inhibition 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 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.\textsuperscript{19} 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
CONTINUOUS

INTERMITTENT

SUBPERIOD 1 second

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.

a 10-second subperiod. Thus, we applied 20-Hz stimulation for 1, 2.5, or 5 seconds but delivered no pulses during the remaining portion of the 10-second subperiod. For example, when we applied 20-Hz stimulation for 2.5 seconds and no pulses during the remaining 7.5 seconds, the mean frequency of pulse delivery was 5 Hz (i.e., 50 pulses/10 sec = 5 Hz). We compared the effects of the intermittent stimulation with the effects of continuous sympathetic stimulation delivered at 2, 5, and 10 Hz. Although the patterns of stimulation differed (i.e., continuous versus intermittent stimulation), the total number of pulses delivered to the sympathetic nerves at a given mean frequency remained constant for equal durations of stimulation. All stimulations were applied for a train duration of 3 minutes.

Drugs

Propranolol (1 mg/kg i.v.) was administered to block β-adrenergic receptors. We considered the blockade of β-adrenergic receptors to be adequate if the cycle length did not change in response to sympathetic stimulation. Phentolamine (2 mg/kg i.v.) was given to block α-adrenergic receptors. To assess whether the α-adrenergic receptor blockade was adequate, we monitored the pressor response to phenylephrine (2–4 μg/kg i.v.) before and after the administration of phentolamine. In the absence of phentolamine, the phenylephrine induced an increase of 43.6 ± 4.7 mm Hg in mean arterial pressure (n = 5). After phentolamine, the phenylephrine elicited no appreciable increase in mean arterial pressure.

Data Analysis

We determined statistical significance by analysis of variance. We considered a significance level of p ≤ 0.05 to be statistically significant. We fitted curves to the data by nonlinear regression analysis (STATGRAPHICS, Statistical Graphics Corp, Rockville, Maryland) and integrated the curves to determine their areas. Data are presented as mean ± SEM.

Results

Representative Experiments

Figure 2 shows a representative example of the cardiac chronotropic responses to our stimulation protocol. Before sympathetic stimulation, the vagi were stimulated for 10 seconds every 2 minutes at a frequency that increased cardiac cycle length by about 100%. After two control vagal stimulations in this example, a train of stimuli (10 Hz for 5 minutes) was delivered to the ansae subclaviae. Cardiac cycle length decreased from 420 to 270 msec during anodal stimulation.

After sympathetic stimulation was discontinued, the vagi were again stimulated for 10 seconds every 2 minutes until the vagally induced chronotropic response returned to control. As shown in Figure 2, the vagally induced cycle-length responses after sympathetic stimulation were initially much smaller than those evoked before sympathetic stimulation. For example, before sympathetic stimulation, vagal stimulation increased cycle length by 460 msec (i.e., cycle length increased from 420 to 880 msec). In contrast, 4 minutes after cessation of sympathetic stimulation the identical vagal stimulation increased cycle length by only 170 msec (i.e., cycle length increased from 410 to 580 msec). Thus, the vagally induced response 4 minutes after sympathetic stimulation was inhibited by 63%. Note that in Figure 2 the chronotropic response to vagal stimulation required about 38 minutes to return to control. We used the stimulation protocol illustrated in Figure 2 to determine the effect of various levels and patterns of sympathetic stimulation on the vagally induced chronotropic responses.

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 \( 100(R_c-R_s)/R_c \), where \( R_c \) is the control vagally induced cycle-length response (before sympathetic stimulation) and \( R_s \) 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

\[ Y = 0.21X^2 \]
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 summated 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 summated inhibition ($p<0.001$, bottom panel). Thus, as the mean frequency of sympathetic stimulation was incremented, both the initial inhibition and the summated 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 summated 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 summated 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 summated 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 summated 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 α- and β-adrenergic-receptor blockade.12,14 Because neuropeptide Y is also released from sympathetic nerve terminals,11,13,18 we sought to determine whether α- or β-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 experimen-
physiological conditions. In support of this conclusion, neuropeptide Y may act to regulate cardiac function under sympathetic activity (e.g., 5 Hz) suggests that neuropeptide Y have not previously been reported in response to low levels of sympathetic stimulation. 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 a steady low frequency (2 Hz) of 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 variates 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 and Allen et al 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 or Allen et al may reflect a tissue or species variation.
Activation of the $\alpha$-adrenergic receptors located on sympathetic nerve terminals inhibits the release of norepinephrine from those nerve endings, whereas blockade of $\alpha$-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 $\alpha$-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 $\alpha$-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 $\alpha$-adrenergic-receptor blockade than under control conditions (Figure 8). Thus, in the heart, the effect of $\alpha$-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 $\alpha$- and $\beta$-adrenergic-receptor blockade, she did not analyze separately the effects of $\alpha$- and $\beta$-adrenergic-receptor antagonists. Our data showed that the inhibition of cardiac vagal effects evoked by sympathetic stimulation is unaffected by $\beta$-adrenergic-receptor blockade, but is significantly enhanced by $\alpha$-adrenergic-receptor blockade (Figure 8). Although the inhibition of the vagal effects on cycle length is nondrenergic in origin, the inhibition is modulated by $\alpha$-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.

Acknowledgments

The authors thank Frank Walters and Harrison Zieske for their expert technical assistance. The propranolol and phentolamine were generously provided by Ayerst Laboratories Inc, New York, and CIBA Pharmaceutical Co, Summit, New Jersey, respectively.

References


Key Words: neuropeptide Y • heart rate • sympathetic • parasympathetic
Neuropeptide Y as a putative modulator of the vagal effects on heart rate.
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Circ Res. 1989;64:882-889
doi: 10.1161/01.RES.64.5.882

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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