

# Inhibition of nitric oxide synthase slows heart rate recovery from cholinergic activation

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**Sears, Claire E., Julia K. Choate, and David J. Paterson.** Inhibition of nitric oxide synthase slows heart rate recovery from cholinergic activation. *J. Appl. Physiol.* 84(5): 1596–1603, 1998.—The role of nitric oxide (NO) in the cholinergic regulation of heart rate (HR) recovery from an aspect of simulated exercise was investigated in atria isolated from guinea pig to test the hypothesis that NO may be involved in the cholinergic antagonism of the positive chronotropic response to adrenergic stimulation. Inhibition of NO synthesis with *N*<sup>G</sup>-monomethyl-L-arginine (L-NMMA, 100  $\mu$ M) significantly slowed the time course of the reduction in HR without affecting the magnitude of the response elicited by bath-applied ACh (100 nM) or vagal nerve stimulation (2 Hz). The half-times ( $t_{1/2}$ ) of responses were  $3.99 \pm 0.41$  s in control vs.  $7.49 \pm 0.68$  s in L-NMMA ( $P < 0.05$ ). This was dependent on prior adrenergic stimulation (norepinephrine, 1  $\mu$ M). The effect of L-NMMA was reversed by L-arginine (1 mM;  $t_{1/2}$   $4.62 \pm 0.39$  s). The calcium-channel antagonist nifedipine (0.2  $\mu$ M) also slowed the kinetics of the reduction in HR caused by vagal nerve stimulation. However, the  $t_{1/2}$  for the reduction in HR with antagonists (2 mM Cs<sup>+</sup> and 1  $\mu$ M ZD-7288) of the hyperpolarization-activated current were significantly faster compared with control. There was no additional effect of L-NMMA or L-NMMA+L-arginine on vagal stimulation in groups treated with nifedipine, Cs<sup>+</sup>, or ZD-7288. We conclude that NO contributes to the cholinergic antagonism of the positive cardiac chronotropic effects of adrenergic stimulation by accelerating the HR response to vagal stimulation. This may involve an interplay between two pacemaking currents (L-type calcium channel current and hyperpolarization-activated current). Whether NO modulates the vagal control of HR recovery from actual exercise remains to be determined.

cardiac; autonomic nervous system; guinea pig

ON CESSATION OF EXERCISE, heart rate (HR) falls toward normal levels caused by a shift in cardiac sympathovagal balance (30). It is well established that vagal nerve stimulation or application of ACh antagonizes the positive cardiac chronotropic effects of adrenergic stimulation to decrease HR, although the mechanism underlying this has not been fully elucidated. Muscarinic cholinergic agonists can reduce HR by direct G protein activation of a potassium current ( $I_{KACh}$ ), (28) and inhibition of the cAMP-stimulated hyperpolarization-activated current ( $I_h$ ) (10). Indirectly, muscarinic cholinergic agonists also decrease HR by inhibition of the  $\beta$ -adrenergic receptor-activated pathway that leads to phosphorylation of the L-type calcium channel ( $I_{CaL}$ ) (19). Recently, the nitric oxide (NO) pathway has been implicated in the parasympathetic control of HR by indirect inhibition of  $I_{CaL}$  (2, 16, 17). This suggests that NO may be involved in the vagal antagonism of the HR response to sympathetic stimulation.

NO is formed from the precursor L-arginine by the enzyme nitric oxide synthase (NOS). In cardiac myocytes, NO is synthesized by NOS III (3) and acts predominantly by binding to the heme moiety of soluble guanylate cyclase to increase the production of guanosine 3',5'-cyclic monophosphate (cGMP; see Ref. 21 for review). In the anesthetized dog (12) and ferret (32), inhibition of NO attenuates the decrease in HR seen with vagal stimulation. The NO pathway has been linked to muscarinic receptor activation by ACh (2), and NO has been shown to affect cardiac channels known to be modulated by autonomic transmitters. In particular, NO decreases  $I_{CaL}$  in adrenergically pre-stimulated frog ventricular cells (25), rabbit sinoatrial node (SAN) (17), and atrioventricular node cells (16), but NO does not affect the regulation of  $I_{KACh}$  by ACh (17). In addition, low concentrations of NO donors can increase  $I_f$  in single rabbit SAN cells and the beating rate of atria isolated from guinea pigs. This effect is virtually abolished by antagonists of  $I_f$  (26). This raises the possibility that modulation of  $I_{CaL}$  and  $I_f$  by NO may be involved in the vagal control of HR after sympathetic activation, particularly during HR recovery from exercise when cardiac sympathovagal activity is transiently high.

To test this hypothesis, we mimicked an aspect of the cardiac autonomic response to exercise and investigated the effects of a NOS inhibitor on the HR response to applied ACh or vagal nerve stimulation in atria isolated from guinea pigs after adrenergic stimulation. This was repeated before and after the addition of antagonists of  $I_{CaL}$  and  $I_f$  to assess the role of these currents in the chronotropic response to vagal nerve stimulation after inhibition of NOS.

## METHODS

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1985) and was performed under British Home Office Project License PPL 30/1133.

### Surgery

Eight male guinea pigs (400–450 g) were killed by cervical dislocation followed by exsanguination. The heart was rapidly removed and placed in warm (35–37°C), oxygenated (95% O<sub>2</sub>-5% CO<sub>2</sub>) Tyrode solution (see *Solutions*) in a Perspex dissecting dish with a Sylgard base. The heart was pinned, and 10 ml of heparinized saline (1,000 U) were injected via the aorta. The lungs were carefully trimmed off, and the ventricles were removed and discarded. Extraneous tissue was removed from around the atria, with particular care being taken not to damage the SAN region. Sutures (6-0 mersilk, Ethicon) were fixed at the lateral edges of the two

atria, a small loop of thread was tied on the right atrium, and a larger loop was tied to the left atrium.

#### Vagal Nerve Dissection

An additional 27 guinea pigs (150–200 g) were killed as described above. The heart was removed with the rib cage and mediasternum. We found that the nerve dissection was more successful with smaller animals. The vagi were separated from the carotid arteries, the left vagus was trimmed away, and the right vagus was isolated and tied. The atria were dissected as above.

#### Measurements

The preparations were then transferred to a preheated ( $37.2 \pm 0.1^\circ\text{C}$ ) organ bath containing 60 ml of continuously oxygenated Tyrode solution. Temperature was monitored by using a Digitron 1408-K temperature gauge, and temperature was controlled by recycling water from a temperature-controlled pump. Tyrode solution was added from a reservoir that contained a glass coil attached to the temperature controller so that any fluid added was already heated. The solutions in the organ bath and in the reservoir were continually bubbled with 95%  $\text{O}_2$ -5%  $\text{CO}_2$ .

The preparations were vertically mounted, with the suture in the right atrium attached to a hook and with the suture in the left atrium tied to a force transducer (HSE F30) that was calibrated before beginning the experiment by using a 10-mN weight. The force transducer was attached to an amplifier, the output was visualized on a Gould ES 1000 recorder, and data were collected via a real-time data-acquisition system (Biopac Systems MP 100) by using Acqknowledge 3.2 software (Macintosh 8500). HR was triggered from contraction and displayed in real time. Data were stored on an optical disk for off-line analysis. The preparations were left for ~90 min until a stable HR ( $\pm 5$  beats/min over 20 min) was achieved.

#### Solutions

The Tyrode solution used throughout the experiment contained (in mM): 120 NaCl, 4 KCl, 2  $\text{MgCl}_2$ , 0.1  $\text{NaH}_2\text{PO}_4$ , 11 glucose, 25  $\text{NaHCO}_3$ , and 1.8  $\text{CaCl}_2$ . The solution was bubbled with 95%  $\text{O}_2$ -5%  $\text{CO}_2$  to give a pH of 7.4.

Norepinephrine (NE) bitartrate (Sigma Chemical) was added from a 1 mM stock solution (frozen in aliquots) to give a concentration of 1  $\mu\text{M}$ . ACh chloride (Sigma Chemical) was added from a 0.1 mM stock solution to give a concentration of 100 nM. *N*<sup>G</sup>-monomethyl-L-arginine (L-NMMA; monoacetate salt, Calbiochem) was added from a 0.1 M stock solution to give a concentration of 100  $\mu\text{M}$ . L-arginine (Sigma Chemical) was added from a 1 M stock solution to give a concentration of 1 mM.

Cesium chloride (Sigma Chemical) was added from a stock solution of 1 M to give a concentration of 2 mM. Nifedipine (Sigma Chemical) was added from a stock solution of 0.01 M to give a concentration of 0.2  $\mu\text{M}$ . Because nifedipine is light sensitive, these experiments were carried out in dark conditions. ZD-7288 was a gift from Zeneca and was added from a 1 mM stock solution to give a concentration of 1  $\mu\text{M}$ .

#### Protocols

*Effect of L-NMMA on the HR response to ACh or vagal stimulation after adrenergic stimulation.* Results obtained with applied transmitters can differ from those obtained by nerve stimulation in terms of their effects on ion currents (6), and results with applied transmitters can often show high

variability compared with nerve stimulation. For these reasons, we tested the effect of L-NMMA on the decline in HR obtained with both applied ACh and with right vagal stimulation in adrenergically stimulated isolated tissue.

ACh (100 nM,  $n = 8$ ) or vagal stimulation (2 Hz, 10 V, 1-ms pulse duration for 15 s,  $n = 8$ ) was added in the continued presence of NE (1  $\mu\text{M}$ ) to maintain background adrenergic stimulation. The amount of vagal stimulation was chosen to mimic the decline of HR seen with applied ACh, and the time period of stimulation meant that desensitization was minimal (24). Hyoscine completely abolished the response to vagal nerve stimulation (data not shown), thus confirming the stimulation of vagal fibers only. The concentration of NE that we used is similar to that seen in the varicosity cleft during sympathetic activation (11). For each condition (control, L-NMMA, and L-NMMA+L-arginine), three vagal stimulations were carried out. Addition of ACh or vagal stimulation after stimulation with NE was repeated after 30 min of exposure to 100  $\mu\text{M}$  L-NMMA. This concentration and time of incubation is acknowledged to result in effective inhibition of NOS without any effect on muscarinic receptors (17). It also has no effect on the baseline HR or contraction. NE, followed by addition of ACh or vagal stimulation, was then repeated again after 20-min exposure to 1 mM L-arginine.

*Effect of L-NMMA on the HR response to ACh or vagal stimulation without adrenergic stimulation.* To investigate whether the effect of L-NMMA on the response to ACh ( $n = 7$ ) or vagal stimulation ( $n = 6$ ) was dependent on prior adrenergic stimulation, we exposed preparations to ACh or vagal stimulation without NE during control, L-NMMA, and L-NMMA+L-arginine.

*Effect of nifedipine on the HR response to vagal stimulation after adrenergic stimulation during L-NMMA.* To assess the contribution of  $I_{\text{CaL}}$  to the HR response to vagal stimulation after NE during L-NMMA, we repeated the protocol described above after a 40-min exposure to 0.2  $\mu\text{M}$  nifedipine ( $n = 8$ ). This concentration was chosen after preliminary experiments, because it was the highest concentration that elicited a stable bradycardia without arresting the preparation. It is also well documented that 0.2  $\mu\text{M}$  nifedipine inhibits  $I_{\text{CaL}}$  (15) and does not affect the transient outward potassium current ( $I_{\text{K}}$ ) (13). Furthermore, at this concentration, dihydropyridines do not affect sodium current,  $I_{\text{K}}$ , or inwardly rectifying potassium current (35).

*Effect of  $\text{Cs}^+$  or ZD-7288 on the HR response to vagal stimulation after adrenergic stimulation during L-NMMA.* To assess the contribution of  $I_{\text{f}}$  to the HR response to vagal stimulation after NE during L-NMMA, the protocol described above was repeated after 20-min exposure to 2 mM  $\text{Cs}^+$  ( $n = 8$ ) or 40-min exposure to 1  $\mu\text{M}$  ZD-7288 ( $n = 3$ ).  $\text{Cs}^+$  (2 mM; see Ref. 8) is widely accepted as a specific antagonist of  $I_{\text{f}}$ , having no effect on  $I_{\text{K}}$  (8), although it may slightly decrease  $I_{\text{KACh}}$  (1). For this reason, another  $I_{\text{f}}$  antagonist (ZD-7288) was used (4).

#### Time Controls

*Effect of applied ACh or vagal stimulation after 30 and 60 min, without L-NMMA and without L-NMMA+L-arginine.* To see whether the effect of L-NMMA on the cholinergic response to HR in adrenergically stimulated hearts was caused by a time-dependent rundown, we performed time controls in both preparations (atria isolated from guinea pigs, with applied transmitter,  $n = 3$ ; and atria isolated from guinea pigs, with intact vagus nerve,  $n = 3$ ). This was done because interventions could not be randomized because of the long incubation period for L-NMMA. Vagal stimulation or bath application of

ACh was undertaken at 0, 30, and 60 min after adrenergic stimulation.

*Statistical Analysis*

Data are presented as means  $\pm$  SE. The kinetics for the HR response to applied ACh were analyzed as a percentage of the maximal HR response to ACh over time, because the data were not well fitted by an exponential curve caused by the nonuniform response to applied ACh (Fig. 1A). The data for vagal stimulation were well fitted by a single exponential (Fig. 2A) and the half-times ( $t_{1/2}$ ) calculated for each condition (control, L-NMMA, and L-NMMA+L-arginine). These were

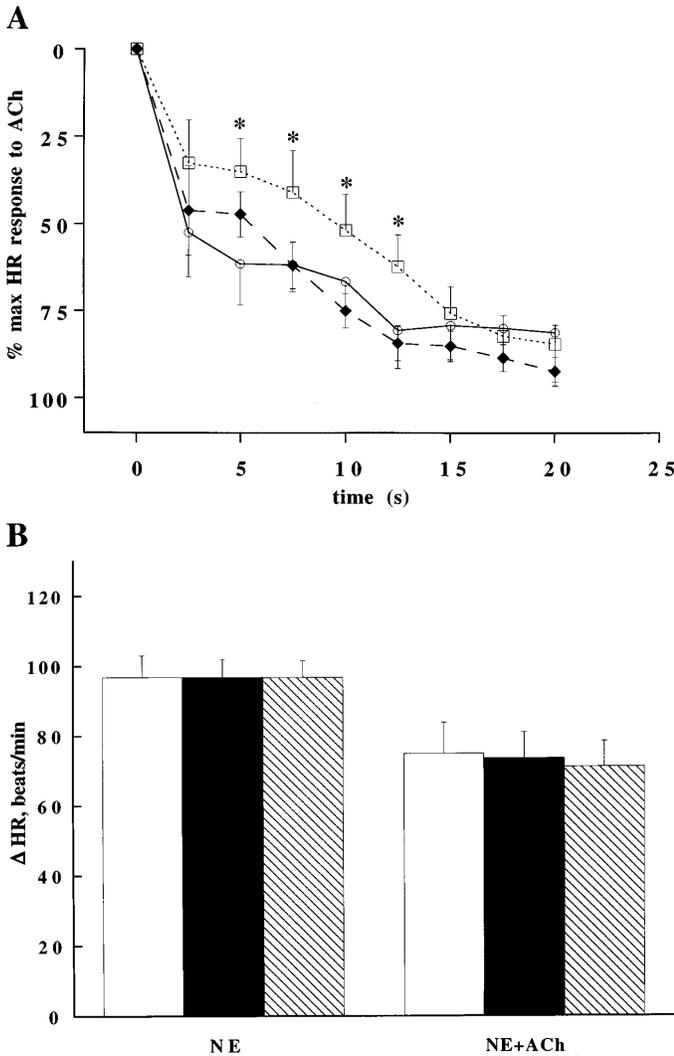


Fig. 1. A: heart rate (HR) response to applied ACh (100 nM) in 8 adrenergically stimulated atria [1  $\mu$ M norepinephrine (NE)];  $\circ$ , control. %Maximal (max) HR response to ACh after 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20 s of HR decrease. %Max HR response to ACh was significantly reduced at 5, 7.5, 10, and 12.5 s (\* $P$  = 0.0071, 0.047, 0.01, and 0.037, respectively, by paired Student's  $t$ -test) in presence of 100  $\mu$ M  $N^G$ -monomethyl-L-arginine (L-NMMA;  $\square$ ). This was reversed by addition of 1 mM L-arginine ( $\blacklozenge$ ). B: HR change ( $\Delta$ HR), in beats/min in 8 atria isolated from guinea pigs, in response to NE (1  $\mu$ M) and 1  $\mu$ M NE+100 nM ACh in each of three conditions: control (open bars), 100  $\mu$ M L-NMMA (solid bars), and 100  $\mu$ M L-NMMA+1 mM L-arginine (hatched bars). The  $\Delta$ HR with NE+ACh was significantly smaller than  $\Delta$ HR with NE alone in all 3 conditions ( $P$  < 0.05, one-way ANOVA). However, there was no significant difference in  $\Delta$ HR for NE or NE+ACh among 3 conditions.

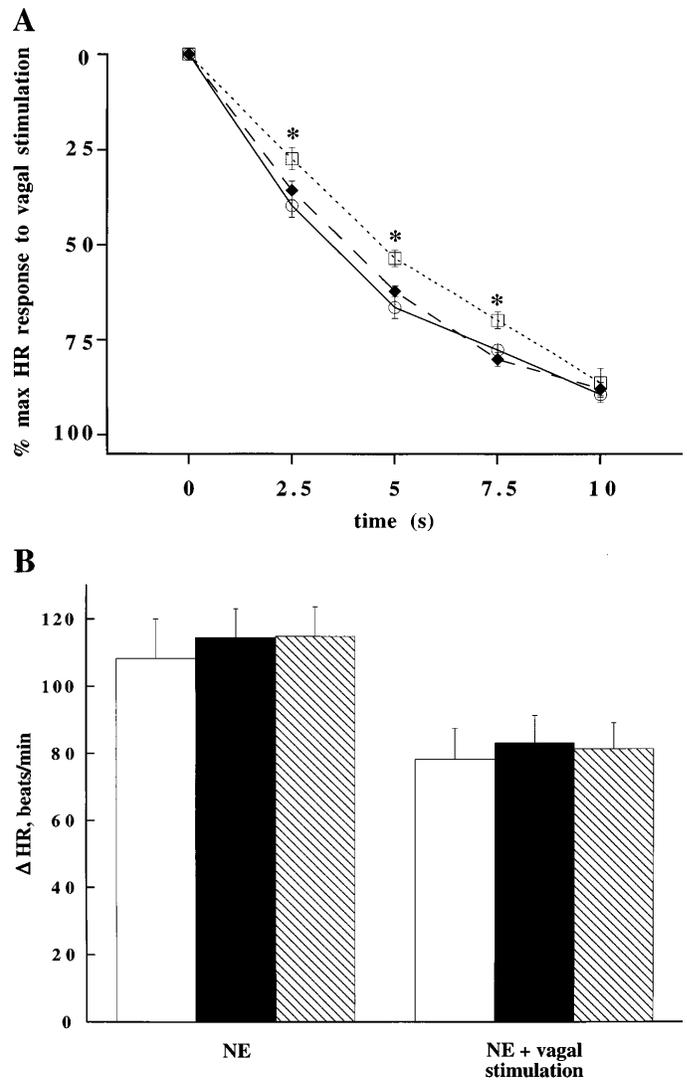


Fig. 2. A: HR response to 2-Hz right vagal stimulation in 8 preadrenergically stimulated atria (1  $\mu$ M NE). Response is shown as %max HR response to vagal stimulation after 2.5, 5, 7.5, and 10 s of stimulation. %Max response was significantly reduced at 2.5, 5, and 7.5 s (\* $P$  = 0.0005, 0.0012, and 0.0035, respectively, paired Student's  $t$ -test) of vagal stimulation in presence of L-NMMA ( $\square$ ).  $\circ$ , Control. This effect was reversed by L-NMMA+L-arginine ( $\blacklozenge$ ). There was a significant increase in  $t_{1/2}$  of decrease in HR with L-NMMA, which was reversed by L-arginine (for values see Table 1, one-way ANOVA). \* Statistical significance compared with control condition ( $P$  < 0.05, paired Student's  $t$ -test). B:  $\Delta$ HR (in beats/min) in 8 atria isolated from guinea pigs in response to NE (1  $\mu$ M) and 1  $\mu$ M NE+vagal stimulation (2 Hz, 10 V, 1-ms duration for 15 s) in each of 3 conditions: control (open bars), 100  $\mu$ M L-NMMA (solid bars), and 100  $\mu$ M L-NMMA+1 mM L-arginine (hatched bars).  $\Delta$ HR with NE+vagal stimulation was significantly smaller than  $\Delta$ HR with NE alone in all 3 conditions ( $P$  < 0.05, one-way ANOVA). There was no significant difference among the 3 conditions in  $\Delta$ HR for NE or NE+vagal stimulation.

compared by using a one-way ANOVA with repeated measures, and post hoc comparison was performed by using Scheffé's test.

This analysis was also used to compare the change in HR between interventions (NE, NE+ACh, or NE+vagal stimulation) and to compare the same intervention in the same atria in different conditions (control, L-NMMA, and L-NMMA+L-arginine). A paired Student's  $t$ -test was used to compare the percentage of HR response over time in different conditions in

the same atria. For comparisons of the same intervention between different sets of atria, an unpaired Student's *t*-test was carried out. Statistical significance was accepted at  $P < 0.05$ .

## RESULTS

### *Effect of L-NMMA on the HR Response to ACh or Vagal Stimulation After Adrenergic Stimulation*

**Applied ACh.** The results were analyzed as the percentage of the maximal HR response to ACh reached at 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20 s of exposure to ACh. L-NMMA decreased the slope of the decline in HR in response to ACh. The percentage of the maximal HR response to ACh was significantly decreased in the presence of L-NMMA ( $P < 0.05$ ) at 5, 7.5, 10, and 12.5 s of the response. This effect was reversed by L-arginine (Fig. 1A) and was dependent on prior adrenergic stimulation.

Neither L-NMMA nor L-arginine affected the baseline HR ( $190.7 \pm 6.9$ ,  $183.3 \pm 5.7$ , and  $189.7 \pm 5.1$  beats/min in control, L-NMMA, and L-NMMA+L-arginine, respectively). The magnitude of the response to NE was the same in all three conditions (Fig. 1B). However, ACh significantly decreased HR in the continued presence of NE (Fig. 1B,  $P < 0.05$ ), although the magnitude of the changes in HR in response to ACh was not significantly different from control in the presence of L-NMMA or L-NMMA+L-arginine [Fig. 1B, change in HR ( $\Delta$ HR) =  $21.9 \pm 6.7$ ,  $18.3 \pm 2.7$ , and  $25.5 \pm 5.7$  beats/min in control, L-NMMA, and L-NMMA+L-arginine, respectively]. No significant change in the kinetics of the decline in HR was noted with ACh alone in control, L-NMMA, or L-arginine (results not shown). There was no significant difference in percentage of the maximal response to ACh reached after 2.5, 5, 7.5, 10, 12.5, and 15 s of exposure, after either 30 or 60 min of the experiment, thus showing the effect of L-NMMA was not caused by time-dependent rundown of the preparation.

**Vagal stimulation.** The decrease in HR in response to vagal stimulation was well fitted by a single-phase exponential (goodness-of-fit regression coefficients:  $0.99 \pm 0.003$  in control,  $0.99 \pm 0.003$  in L-NMMA, and  $0.99 \pm 0.004$  in L-NMMA+L-arginine), and the  $t_{1/2}$  that were calculated for decrease in HR (Table 1). L-NMMA significantly slowed the decline of HR in response to vagal stimulation (Fig. 2A). The  $t_{1/2}$  was significantly increased from control with L-NMMA ( $3.99 \pm 0.41$  vs.  $7.49 \pm 0.68$  s, respectively;  $P < 0.05$ ), and the response was reversed by L-arginine ( $t_{1/2} = 4.62 \pm 0.39$  s). These results were also analyzed as the percentage of the maximal HR response to vagal stimulation reached at 2.5, 5, 7.5, and 10 s (Fig. 2A). The shorter time period of analysis, compared with that for applied ACh, is caused by the faster decrease in HR seen with vagal stimulation. The percent maximal response was significantly reduced ( $P < 0.05$ ) at 2.5, 5, and 7.5 s in the presence of L-NMMA. There was no effect of L-NMMA on the HR response to vagal stimulation without prestimulation by NE ( $t_{1/2} = 1.71 \pm 0.27$

**Table 1.** *Effect of nifedipine, cesium, or ZD-7288 on  $t_{1/2}$  for time course of vagally induced decrease in heart rate in L-NMMA and L-arginine*

Group	Control	L-NMMA	L-NMMA + L-Arginine
Control	$3.99 \pm 0.41$	$7.49 \pm 0.39^*$	$4.62 \pm 0.39$
Nifedipine	$5.30 \pm 1.31$	$6.64 \pm 0.29$	$5.39 \pm 1.36$
Cesium	$1.80 \pm 0.34^\dagger$	$1.90 \pm 0.36$	$1.28 \pm 0.12$
ZD-7288	$2.40 \pm 0.30^\dagger$	$2.30 \pm 1.10$	2.89

Values are means  $\pm$  SE of  $t_{1/2}$  (in s) for time course of vagally induced decrease in heart rate (2 Hz, 10 V, 1-ms pulse duration for 15 s) in control conditions in presence of 100  $\mu$ M L-NMMA and in 100  $\mu$ M L-NMMA + 1 mM L-arginine in isolated guinea pig atria. Results shown for 4 sets of experiments: control hearts ( $n=8$ ), with 0.2  $\mu$ M nifedipine ( $n=8$ ), 2 mM Cs<sup>+</sup> ( $n=8$ ), and 1  $\mu$ M ZD-7288 ( $n=3$  for Control and L-NMMA conditions;  $n=2$  for L-NMMA + L-arginine condition). Half-times were generated from decay in rate over time by fitting a monoexponential curve (goodness-of-fit regression coefficient  $0.99 \pm 0.003$  in control;  $0.99 \pm 0.003$  in nifedipine;  $0.98 \pm 0.01$  in Cs<sup>+</sup>; and  $0.99 \pm 0.001$  in ZD-7288). The  $t_{1/2}$  was significantly increased ( $P < 0.01$ , one-way ANOVA) in control experiments in presence of L-NMMA; this effect was reversed by L-arginine. There was no significant increase in  $t_{1/2}$  in nifedipine, Cs<sup>+</sup> or ZD-7288 with L-NMMA. Half-time in control condition was slower with nifedipine but significantly faster with Cs<sup>+</sup> or with ZD-7288 ( $P=0.004$  and  $0.025$ , respectively). \* Value within a group that was significantly different from its own control (one-way ANOVA).  $\dagger$  Value between groups that was significantly different from control group (unpaired Student's *t*-test).

and  $2.11 \pm 0.29$  s in control and in L-NMMA, respectively;  $P = 0.21$ ,  $n = 6$ ).

L-NMMA did not affect the baseline HR ( $215.2 \pm 8.6$ ,  $203.8 \pm 8.8$ , and  $203.5 \pm 8.8$  beats/min in control, L-NMMA, and L-NMMA+L-arginine, respectively). The change in HR with NE was also not significantly different in control, L-NMMA, or L-NMMA+L-arginine ( $\Delta$ HR =  $108.3 \pm 11.1$ ,  $114.6 \pm 8.2$ , and  $114.9 \pm 8.8$  beats/min, respectively; Fig. 2A). However, vagal stimulation significantly decreased HR in the continued presence of NE (Fig. 2B;  $P < 0.05$ ). The magnitude of the response to vagal stimulation was not significantly different in control, with L-NMMA, or with L-NMMA+L-arginine ( $\Delta$ HR =  $36.2 \pm 5.4$ ,  $30.2 \pm 3.7$ , and  $29.8 \pm 3.3$  beats/min, respectively; Fig. 2B). In summary, L-NMMA significantly slowed the kinetics of the HR response to bath-applied ACh or to vagal stimulation, although the magnitude of the response was not altered.

### *Effect of L-NMMA on the HR Response to Vagal Stimulation After Adrenergic Stimulation in the Presence of Nifedipine*

There was no effect of L-NMMA on the kinetics of the response to vagal stimulation after adrenergic stimulation in the presence of nifedipine (0.2  $\mu$ M; Fig. 3). The  $t_{1/2}$  for HR decrease in the nifedipine-treated group was slower than that in the control; however, this was not statistically significant (Table 1,  $P = 0.62$ ), although the response to nifedipine was significantly different at  $t = 5$  s in L-NMMA (unpaired Student's *t*-test,  $P < 0.05$ ).

Nifedipine decreased the basal HR from  $215.2 \pm 8.6$  to  $173.4 \pm 10.3$  beats/min ( $P < 0.05$ ). The increase in HR with NE was not significantly different from that

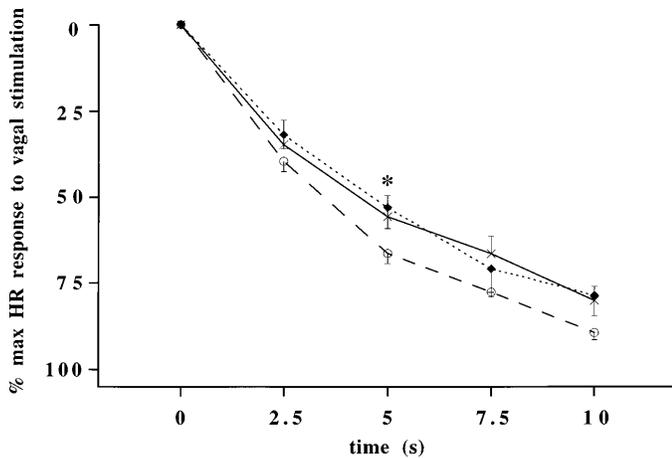


Fig. 3. HR response to right vagal stimulation (2 Hz, 10 V, 1-ms pulse duration, 15-s stimulation) in preadrenergically stimulated (1  $\mu$ M NE) atria. %Max HR response to vagal stimulation is shown after 2.5, 5, 7.5, and 10 s of stimulation for control ( $\circ$ ), nifedipine (0.2  $\mu$ M;  $\blacklozenge$ ), and nifedipine+100  $\mu$ M L-NMMA ( $\times$ );  $n = 8$  for each group. Control data are from group of atria represented in Fig. 2. Statistical comparison was via an unpaired  $t$ -test. There was no significant difference between  $t_{1/2}$  in the 2 conditions (nifedipine and nifedipine+100  $\mu$ M L-NMMA). For computed values see Table 1. \*Statistically significant difference in %max HR response to vagal stimulation at  $t = 5$  s in nifedipine+L-NMMA compared with control (unpaired  $t$ -test,  $P < 0.05$ ).

seen in control ( $\Delta$ HR =  $104.5 \pm 12.5$  vs.  $108.3 \pm 11.1$  beats/min in nifedipine vs. control, respectively). The change in HR with vagal stimulation was not significantly different from that seen in control conditions ( $\Delta$ HR =  $45.4 \pm 7.6$  vs.  $36.2 \pm 5.4$  beats/min in nifedipine vs. control, respectively).

#### Effect of L-NMMA on the HR Response to Vagal Stimulation After Adrenergic Stimulation in the Presence of $I_f$ Antagonists

There was no effect of L-NMMA on the kinetics of the decline in HR caused by vagal stimulation in hearts adrenergically stimulated with  $\text{Cs}^+$  (2 mM; Fig. 4). However, the  $t_{1/2}$  for HR decrease in  $\text{Cs}^+$  alone was significantly faster than that in control (Table 1).

$\text{Cs}^+$  caused the basal HR to decline from  $215.2 \pm 8.6$  to  $165.4 \pm 5.4$  beats/min ( $P < 0.05$ ). This decrease with  $\text{Cs}^+$  was not significantly different from that seen in nifedipine-treated groups. The decrease in HR with vagal stimulation in  $\text{Cs}^+$  was not significantly different from that in control ( $\Delta$ HR =  $36.2 \pm 5.4$  vs.  $30.5 \pm 4.6$  beats/min in control vs.  $\text{Cs}^+$ , respectively). However, the increase in HR with NE in  $\text{Cs}^+$  was significantly smaller than that seen in control ( $\Delta$ HR =  $38.8 \pm 4.9$  vs.  $108.3 \pm 11.1$  beats/min in  $\text{Cs}^+$ -treated groups compared with control;  $P < 0.05$ ).

ZD-7288 (1  $\mu$ M) gave results qualitatively similar to those seen with  $\text{Cs}^+$ . There was no effect of L-NMMA on the kinetics of the decline of HR with vagal stimulation in the presence of ZD-7288. However, the  $t_{1/2}$  of HR decrease in ZD-7288 alone and with L-NMMA was faster than that seen in control (Table 1). The basal HR was  $98.1 \pm 4.3$  vs.  $215.2 \pm 8.6$  beats/min in ZD-7288 vs. the control experiments, respectively. The increase in

HR with NE with ZD-7288 was significantly smaller than that in the control experiments ( $\Delta$ HR =  $49.1 \pm 1.3$  vs.  $108.3 \pm 11.1$  beats/min, ZD-7288 vs. control, respectively). The change in HR with vagal stimulation was not significantly different from that in control ( $\Delta$ HR =  $21.9 \pm 6.7$  vs.  $36.2 \pm 5.4$  beats/min in ZD-7288-treated compared with control group, respectively).

#### DISCUSSION

The role of the NO pathway in the autonomic control of HR has received relatively little attention. Studies of single cells suggest that the muscarinic receptor-activated NO pathway decreases HR by antagonizing adrenergically stimulated  $I_{\text{CaL}}$  (16, 17, 25): the so-called "indirect effect" (17, 18). We provide functional evidence for the following effects. 1) Inhibition of NO synthesis by L-NMMA slows the time course of the reduction in HR caused by bath-applied ACh or vagal nerve stimulation in adrenergically stimulated atria isolated from guinea pigs. This effect was reversed by L-arginine. 2) The magnitude of the cholinergic-induced reduction in HR was unaffected by L-NMMA. 3) Nifedipine slowed the kinetics of the vagal decrease in HR, with no further effect of L-NMMA. This is consistent with the hypothesis that NO modulation of HR may be via an indirect effect on  $I_{\text{CaL}}$  (17). However, with application of  $\text{Cs}^+$  or ZD-7288, the kinetics were faster than in control. There was also no effect of L-NMMA on this response.  $I_f$  stimulation by NO (26) might, therefore, attenuate the decrease in HR caused by vagal activation. Taken together, these results suggest that NO

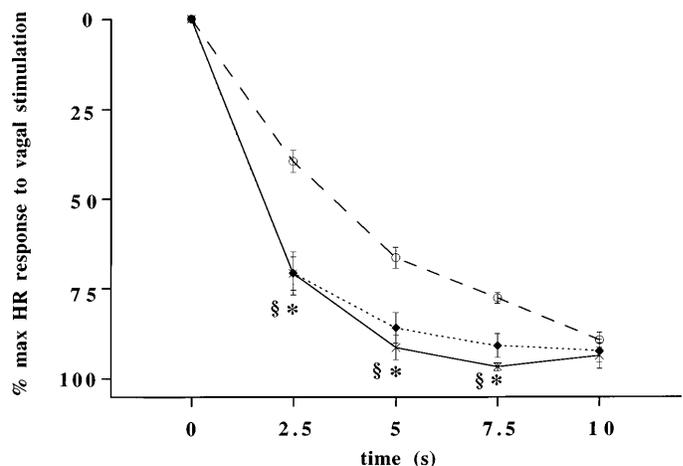


Fig. 4. %Max HR response to right vagal stimulation (2 Hz, 10 V, 1-ms pulse duration, 15-s stimulation) in preadrenergically stimulated (1  $\mu$ M NE) atria. Results are shown for control ( $\circ$ ), 2 mM  $\text{Cs}^+$  ( $\blacklozenge$ ), and 2 mM  $\text{Cs}^+$ +100  $\mu$ M L-NMMA ( $\times$ );  $n = 8$  for each group. Control data are from group of atria represented in Fig. 2. Results were analyzed as %max HR response to vagal stimulation after 2.5, 5, 7.5, and 10 s of stimulation. Slope of decline of HR was increased in presence of  $\text{Cs}^+$ . \*Statistically significant increase in %max HR response occurred at 2.5, 5, and 7.5 s in  $\text{Cs}^+$ -stimulated group compared with control, unpaired  $t$ -test;  $P < 0.05$ . §§Statistically significant difference at 2.5, 5, and 7.5 s in the presence of  $\text{Cs}^+$ +L-NMMA;  $P < 0.05$ . There was no significant difference between slopes in  $\text{Cs}^+$  or  $\text{Cs}^+$ +L-NMMA (for  $t_{1/2}$  see Table 1), but significant decrease occurred in  $t_{1/2}$  in  $\text{Cs}^+$  compared with  $t_{1/2}$  in control.

may be involved in an interplay between  $I_{CaL}$  and  $I_f$  in the autonomic regulation of HR.

### *NO and Autonomic Regulation of Pacemaking*

In cultured, spontaneously beating, neonatal rat ventricular myocytes, inhibition of NO synthesis blocked the negative chronotropic effect of carbachol and analogs of cGMP (2). This is thought to result primarily from the NO pathway reducing  $I_{CaL}$ , because cholinergic inhibition of adrenergically stimulated  $I_{CaL}$  in isolated SAN and atrioventricular node cells is abolished by L-NMMA (16, 17). In anesthetized dogs (12) and ferrets (7), NOS inhibitors slowed the time course of the decrease in HR and reduced the magnitude of the decrease in HR in response to vagal stimulation. Interestingly, both studies (7, 12) demonstrated this effect with no preadrenergic stimulation. In contrast, we have found that L-NMMA, while also slowing the time course of the decrease in HR during cholinergic modulation, did not change the magnitude of the response. This suggests that other current systems are major contributors to the decrease in HR with cholinergic activation (e.g.,  $I_{KA Ch}$ ).

The response to L-NMMA was also dependent on prior adrenergic activation. This observation is consistent with those of others (16, 17) who have shown that L-NMMA only inhibits the action of cholinergic agonists in SAN cells when  $I_{CaL}$  is prestimulated with isoprenaline. The need for prior adrenergic stimulation could be caused by a  $Ca^{2+}$ -dependent activation of the NOS enzyme (14) or by the cholinergically activated NO inhibition of  $I_{CaL}$  occurring only when the current is increased by catecholamines. Furthermore, in our preparations and in those of Elvan et al. (12), there was no effect of L-NMMA on basal HR, indicating no role for NO production in the control of unstimulated HR. We also observed that L-NMMA did not alter the magnitude of the HR response to NE, whereas inhibition of NOS augmented the response to adrenergic stimulation in anesthetized dogs (20).

Our results suggest that there may be an interplay between  $I_{CaL}$  and  $I_f$  during cholinergic activation of the NO pathway. Nifedipine,  $Cs^+$ , and ZD-7288 all reduced the baseline HR; however, the magnitude of the vagal stimulation was not significantly altered by these inhibitors. Nifedipine slowed the time course of the decrease in HR caused by vagal stimulation. The effect of L-NMMA on the kinetics of the HR response was absent in the presence of nifedipine, suggesting that inhibition of  $I_{CaL}$  is involved in the decrease in HR caused by vagal activation of the NO pathway. Even though  $I_f$  is inhibited by ACh in SAN cells (10), in our experiments with  $I_f$  antagonists, 2 mM  $Cs^+$  (8), or 1  $\mu$ M ZD-7288 (4), the kinetics of the vagally induced decrease in HR were significantly faster. A compatible finding was reported by Boyett et al. (5), who noted that inhibition of  $I_f$  with UL-FS49 potentiated the negative chronotropic effect of vagal stimulation in a small multicellular preparation from the rabbit SAN (see Fig. 4 in Ref. 5). Interestingly, cGMP stimulates  $I_f$  in excised patches from SAN cells (9), and NO donors can cause an increase in HR by

stimulation of  $I_f$  via the NO-cGMP pathway (26). This latter effect is virtually blocked by  $Cs^+$  or ZD-7288 (26). These observations are consistent with a faster decline in HR during vagal stimulation of the NO pathway in the presence of  $I_f$  antagonists and may be related to direct vagal activation of  $I_{KA Ch}$  (34). Moreover, activation of  $I_{KA Ch}$  by cholinergic agonists has been shown to be unaffected by NOS inhibitors (17). This may explain why we did not observe an effect of L-NMMA on the kinetics of the HR response during vagal stimulation with  $Cs^+$  or ZD-7288. Another possibility is that, with cholinergic stimulation, the magnitude of  $I_f$  is increased because of hyperpolarization caused by activation of  $I_{KA Ch}$ . However, there is little convincing evidence that low frequencies of vagal stimulation result in a significant hyperpolarization (6).

NOSs are expressed in cardiac myocytes (3), endothelial cells (22), and adrenergic and cholinergic pre- and postganglionic neurons (see Ref. 23 for review), although the relative importance of the specific cell type involved in the production of NO in the heart is not known. In our preparation of atria with intact vagus isolated from guinea pigs, it is likely that NOS-containing neurons, as well as muscarinic receptor-activated NOS, may be an important source for the synthesis of NO, given that both sources would have been inhibited by the nonspecific NOS inhibitor L-NMMA (29). In our preparations, coincubation of L-NMMA with the NOS endogenous substrate L-arginine restored the cholinergic-induced decrease in HR back to the control response. This result indicates that the effects of L-NMMA other than those of NOS inhibition are not responsible for our results. Similarly, L-NMMA with L-arginine restored the carbamylcholine-induced decrease in  $I_{CaL}$  in SAN cells, indicating that L-NMMA does not interfere directly with the muscarinic effects of cholinergic inhibition of  $I_{CaL}$  (17).

### *Limitations of Study to HR Recovery from Exercise*

It is widely accepted that modulation of cardiac sympathovagal balance is predominantly responsible for HR recovery from exercise (30), although other factors may contribute (e.g., temperature and mechanical modulation of SAN). On cessation of exercise, vagal rebound drops HR, even though catecholamines remain transiently high (27). Moreover, the kinetics of this recovery are markedly slowed in (vagotomized and sympathectomized) heart transplant patients (31) and after administration of atropine in normal subjects (30).

Extrapolation of our results to an in vivo preparation is limited by the nature of our Tyrode-perfused isolated atrial preparation from guinea pigs. In the conscious animal, other factors (both neural and circulating) might affect the HR response of the heart to NO. However, to study the role of endogenous NO in the cholinergic control of HR, it was necessary to delimit our protocol to the isolated cardiac preparations with and without vagal innervation.

Our model reflects only a component of the autonomic response of the HR recovery from simulated

exercise. Vagal stimulation for 15 s at 2 Hz shows minimal desensitization of muscarinic receptors to ACh (24); therefore, longer periods of stimulation were inappropriate. Given the nonspecific nature of L-NMMA, we cannot speculate on the source of NO. Additional experiments that use more isoform-specific NOS inhibitors would help identify this. The physiological significance of our results remain to be determined in awake humans, but it is conceivable that a NO-dependent pathway may play a small role in decreasing HR on cessation of exercise when cardiac sympathovagal activity is transiently high.

In summary, we have shown a functional role for the NO pathway in the control of HR in a mammalian heart. This pathway contributes to the cholinergic antagonism of the positive cardiac chronotropic effects of adrenergic stimulation by slowing the kinetics of the HR response to vagal stimulation without affecting the magnitude of the decrease in HR. Our results suggest that the NO pathway may have an opposing action on two pacemaker currents. It may act to inhibit  $I_{CaL}$  to slow the HR, but it may also stimulate  $I_f$  thereby attenuating the rapid decrease in HR caused by cholinergic activation. It remains to be determined whether NO modulates the vagal control of HR recovery from actual exercise.

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