Cardiac enkephalins attenuate vagal bradycardia: interactions with NOS-1-cGMP systems in canine sinoatrial node

Martin Farias III, Keith Jackson, Michael Johnson, and James L. Caffrey

Department of Integrative Physiology, Cardiovascular Research Institute, University of North Texas Health Science Center, Fort Worth, Texas 76107

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Farias III, Martin, Keith Jackson, Michael Johnson, and James L. Caffrey. Cardiac enkephalins attenuate vagal bradycardia: interactions with NOS-1-cGMP systems in canine sinoatrial node. Am J Physiol Heart Circ Physiol 285: H2001–H2012, 2003. First published July 24, 2003; 10.1152/ajpheart.00275.2003.—Endogenous opioids and nitric oxide (NO) are recognized modulators of cardiac function. Enkephalins and inhibitors of NO synthase (NOS) both produce similar interruptions in the vagal control of heart rate. This study was conducted to test the hypothesis that NO systems within the canine sinoatrial (SA) node facilitate local vagal transmission and that the endogenous enkephalin methionine-enkephalin-arginine-phenylalanine (MEAP) attenuates vagal bradycardia by interrupting the NOS-cGMP pathway. Microdialysis probes were inserted into the SA node, and they were perfused with nonselective (N-nitro-l-arginine methyl ester) and neuronal (7-nitroindazole) NOS inhibitors. The right vagus nerve was stimulated and both inhibitors gradually attenuated the resulting vagal bradycardia. The specificity of these inhibitions was verified by an equally gradual reversal of the inhibition with an excess of the NOS substrate l-arginine. Introduction of MEAP into the nodal interstitium produced a quickly developing but quantitatively similar interruption of vagal bradycardia that was also slowly reversed by the addition of l-arginine and not by D-arginine. Additional support for convergence of opioid and NO pathways was provided when the vagolytic effects of MEAP were also reversed by the addition of the NO donor S-nitroso-N-acetyl-penicillamine, the protein kinase G activator 8-bromo-cGMP, or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. MEAP and 7-nitroindazole were individually combined with the direct acting muscarinic agonist carbachol was substituted for direct nerve stimulation at a site proximal to the muscarinic receptors resident on the pacemaker cells (5, 14). Extensive agonist/antagonist profiles indicated that the receptors responsible for these observations are δ-opioid receptors (15, 20). These observations were consistent with the hypothesis that prejunctional δ-opioid receptors suppress acetylcholine release from local vagal nerve terminals within the SA node.

NO also moderated parasympathetic function (8, 10, 11, 13, 17–19, 23, 24). Interruption of the NO-cGMP pathway attenuated vagal bradycardia in several experimental models (8, 10, 11, 13, 17–19). NO synthase (NOS) inhibitors reduced the negative chronotropic response to vagal stimulation in both isolated tissue and whole animal model systems (10, 12, 13, 17, 19). A selective neuronal NOS (NOS-1) inhibitor produced a qualitatively similar vagolytic effect suggesting that the affected enzyme was resident within the network of intrinsic and extrinsic cardiac nerves (18). NOS-1 was immunocytochemically localized in choline acetyltransferase-positive cells within the atria and NOS inhibitors were ineffective when the mixed cholinergic agonist carbachol was substituted for direct nerve stimulation (7, 18). These findings suggested that NO improves vagal control of heart rate by facilitating the local release of acetylcholine. None of these studies, however, distinguished between actions within the node or within the nearby intracardiac parasympathetic ganglia.

NO may improve vagal transmission in the heart indirectly by reducing the degradation of neuronal cAMP. The accumulating cyclic nucleotide activates protein kinase A, increases Ca2+ influx, and thus facilitates vesicular neurotransmitter release. In this proposed mechanism, NO initially raises cGMP by increasing guanylyl cyclase activity. cGMP activates cGMP-dependent protein kinase (PKG), which then slows the rate of cAMP hydrolysis by suppressing phosphodiesterase (PDE) activity (PDE-3). This sequence of events ultimately leads to an accumulation of cAMP (17, 18).

ENDOGENOUS ENKEPHALINS and nitric oxide (NO) are gaining recognition for their effects on cardiac parasympathetic function (4–11, 13–15, 17–21). The endogenous opioid methionine-enkephalin-arginine-phenylalanine (MEAP) is a potent inhibitor of vagally mediated bradycardia. In the canine heart, MEAP interrupted vagal transmission within the sinoatrial (SA) node at a site proximal to the muscarinic receptors resident on the pacemaker cells (5, 14). Extensive agonist/antagonist profiles indicated that the receptors responsible for these observations are δ-opioid receptors (15, 20). These observations were consistent with the hypothesis that prejunctional δ-opioid receptors suppress acetylcholine release from local vagal nerve terminals within the SA node.

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Interactions between opioids and the NO-cGMP pathway have been reported in several tissues (2, 3, 12, 22, 27). The local administration of the δ-1-selective opioid [d-Pen2,5]enkephalin (DPDPE) into the mouse brain and spinal cord decreased NOS activity (2, 3). δ-Opioids also reduced NO release in selected vascular endothelial and intestinal model systems (22, 27). Collectively, these findings suggest that opioids can interact with a variety of NO-generating systems. Similarities between the vagolytic effects of enkephalin and NOS inhibitors suggested that the opioids might exert their vagolytic activity in heart by interrupting the NOS-cGMP pathway.

This study was conducted to test the hypothesis that NO systems within the canine SA node facilitate local vagal transmission and that the endogenous enkephalins like MEAP attenuate vagal bradycardia by interrupting the NOS-cGMP pathway. In the studies that follow, the gross anatomic location and the characteristics of the nodal NOS activity were evaluated in the SA node by microdialysis. Two strategies were employed. First, the vagolytic effects of NOS inhibitors and MEAP were compared with respect to time course, magnitude, specificity, and interaction with cholinergic agonists. Second, the points of interaction or convergence of NO and opioid mechanisms were tested by evaluating the ability of selected components in the NOS-cGMP pathway to override the vagolytic effect of MEAP.

METHODS

Experiments conformed to the National Institutes of Health Guide for the Care and the Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1985).

Surgical preparation. Forty-three mongrel dogs were anesthetized with pentobarbital sodium (32.5 mg/kg), intubated, and mechanically ventilated with room air. Fluid-filled catheters were inserted into the femoral artery and vein and then advanced into the descending aorta and inferior vena cava, respectively. The arterial line was attached to a Statham PD 23XL pressure transducer to monitor heart rate and blood pressure continuously (PowerLab). The venous line was used to administer additional anesthetic as needed. Arterial blood gases were monitored with a blood gas analyzer (Instrumentation Laboratories) and the PO2 (90–120 mmHg), pH (7.35–7.45), and PCO2 (35–45 mmHg) were maintained within physiological limits by oxygen supplementation, bicarbonate administration, or by adjustment of the minute volume, respectively.

The right and left vagus nerves were isolated through a midline surgical incision and tied off securely with umbilical tape and the nerves were returned to the cervical compartment for later retrieval. A single dose of succinylcholine (50 μg/kg) was administered intravenously to temporarily reduce involuntary muscle movements during the 10–15 min required for the electroosmotic incision of the right thorax and the costal-ternal cartilage for ribs 2–5. The pericardium was opened and the upper pericardial margins were sutured to the body wall to support and stabilize the heart.

Because NO reportedly also reduces norepinephrine release from cardiac sympathetic nerves, the primary cardiac sympathetic nerves were severed bilaterally (26). The sympathetic nerves were isolated as they exit the stellate ganglia (ansa subclavia). In each case, their functional identity was verified by a brief stimulation of the nerve (1 Hz, 15 V, 15 s) to observe an increased heart rate and/or pulse pressure. The nerves were then ligated with suture and severed to eliminate complicating interactions between NOS and adrenergic systems.

A 27-gauge stainless steel cannula was used to introduce a linear microdialysis probe into the center of the sinoatrial node parallel to its long axis. The cannula with the dialysis line inside was inserted into the node. The cannula was withdrawn and the dialysis window was positioned within the nodal tissue. After the probe was positioned, the line was perfused with saline for 60 min to allow for the equilibration of interstitial conditions around the newly inserted probe. At the end of each experiment, norepinephrine (1 × 10−9 mol/μl) was briefly introduced into the microdialysis probe to confirm the accurate placement of the probe within the SA node. A brisk increase in heart rate provided functional verification of the nodal location. Prior studies determined that deliberate repositioning of the probe as little as 2 mm lateral to the node eliminated the norepinephrine-mediated tachycardia. The microdialysis delivery microstructured angle 1-cm length of dialysis fiber from a Clirans TAF08 (Asahi Medical) artificial kidney (200 μm ID, 220 μm OD) and hollow silica (SGE; Austin, TX) inflow and outflow tubes (120 μm ID, 170 μm OD). The dialysis tubing permits molecules with a molecular mass of 35,000 kDa or less to cross from the lumen into the nodal interstitium. This technique allows the precise introduction of agents directly into the nodal interstitium for extended periods without provoking complicating systemic reflexes.

Statistical methods. All data were analyzed with repeated-measures ANOVA, and post hoc analyses were performed with Tukey’s test for multiple comparisons. Differences determined to occur by chance with a P < 0.05 were deemed statistically significant.

Protocol 1. The purpose of this protocol was to test whether NOS is an integral component of vagal transmission within the SA node. Nonselective Nω-nitro-L-arginine methyl ester (L-NAME, Sigma; St. Louis, MO) or NOS-1 selective 7-nitroindazole (Sigma), NOS inhibitors, were used to evaluate total and neuronal NOS contributions during vagal bradycardia. Dose responses were determined for each inhibitor in separate groups of animals. Each dose of each inhibitor (0.5, 5, and 15 × 10−9 mol/min) was added to the dialysis inflow and perfused for 60 min. The right vagus nerve was stimulated for 15 s each at 2 and 4 Hz (5 ms, 15 V). One minute and forty-five seconds was allowed for recovery between the two sequential stimulation frequencies. These vagal/heart rate-frequency responses were determined before and then after 15, 30, 45, and 60 min of exposure to the inhibitor. After 1 h, the inhibitor was combined with a molar excess of the NOS substrate L-arginine (50 × 10−9 mol/min) for another 60 min to verify that the effects of L-NAME and 7-nitroindazole resulted from competitive NOS inhibition. Vagal stimulations were repeated at 15-min intervals. L-Arginine and the NOS inhibitor were washed out for 15 min and baseline vagal responses were verified. The protocol was repeated as just described above for the next two doses of inhibitor.

 Protocol 2. These protocols were designed to test whether enhancing the NOS-cGMP pathway in the SA node reverses the vagolytic effect of MEAP and to discern where in the proposed pathway NO and the opioids are most likely to interact. Microdialysis probes were inserted into the SA node and after 60 min of equilibration the probe was perfused with saline for another 60 min. Two-point vagal frequency responses were determined as described in protocol 1 at 15-min
Table 1. Baseline hemodynamic variables

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Treatment</th>
<th>Washout</th>
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<td></td>
<td>HR</td>
<td>MAP</td>
<td>HR</td>
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<tr>
<td>MEAP</td>
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<td>98 ± 9</td>
<td>120 ± 7</td>
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<td>L-NAME</td>
<td>98 ± 6</td>
<td>100 ± 5</td>
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<tr>
<td>7-NT</td>
<td>117 ± 5</td>
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<td>118 ± 8</td>
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<td>112 ± 7</td>
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</tr>
<tr>
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<td>108 ± 5</td>
<td>115 ± 8</td>
</tr>
<tr>
<td>IBMX</td>
<td>116 ± 7</td>
<td>112 ± 7</td>
<td>113 ± 6</td>
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Values are means ± SE. HR, heart rate (in beats/min); MAP, mean arterial pressure (in mmHg); MEAP, methionine-enkephalin-arginine-phenylalanine; L-NAME, Nω-nitro-L-arginine methyl ester; 7-NT, 7-nitroindazole; L-Arg, L-arginine; SNAP, S-nitroso-N-acetylpenicillamine; d-Arg, d-arginine; and IBMX, isobutyl-methylxanthine. Shown are the resting HR and blood pressure after the nodal perfusate was returned to vehicle at the end of each experiment.

Fig. 1. A: heat rate [in beats/min (bpm)]/frequency response during right vagal nerve stimulation at 60 min during the nodal delivery of saline vehicle, the general nitric oxide (NO) synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) (5 × 10⁻⁹ mol/min) alone, and L-NAME combined with L-arginine (L-Arg, 50 × 10⁻⁹ mol/min). B: heart rate/frequency responses during right vagal nerve stimulations with increasing doses of L-NAME and with each dose combined with L-Arg (50 × 10⁻⁹ mol/min, 60 min). In an effort to save space, only the 60-min value for the combination with L-Arg is shown. Vagal stimulations were performed at 15-min intervals during the 60-min treatments. *P < 0.05, significantly different from control. Values are means ± SE, n = 5.
10⁻⁹ mol/min) was introduced into the dialysis inflow and perfused for 5 min. After 5 min, the vagus was retested to verify that the vagolytic effect of intranodal MEAP was intact in these animals. MEAP was washed out and methacholine (1 × 10⁻⁷ mol/min) and MEAP (5 × 10⁻⁹ mol/min) were combined in the dialysis inflow. The heart rate was recorded at 5-min intervals for 30 min. In separate experiments, the same protocol was performed to evaluate postjunctional interactions between methacholine and the NOS-1 inhibitor 7-nitroindazole.

RESULTS

Forty-three dogs were assigned to various protocols employing enkephalin, NOS inhibitors, and various components of the NO-mediated cyclic nucleotide second messenger system. Table 1 represents the resting cardiovascular parameters for all animals across all treatments. Although there was a substantial range in initial values, the resting heart rate and blood pressure were not different among groups. Resting heart rate and blood pressure were also statistically unaltered by any of the treatments perfused into the SA node via cardiac microdialysis, regardless of dose.

L-NAME dose response. The purpose of this portion of protocol 1 was to determine whether NOS in the sinoatrial node was a requisite part of vagally mediated bradycardia. Control vagal stimulations during vehicle infusion produced frequency-dependent two-step declines in heart rate that remained consistent throughout the 6-h experimental protocol. When L-NAME (5 × 10⁻⁹ mol/min; n = 5) was introduced into the nodal interstitium, vagally mediated bradycardia gradually declined by 50%, with the maximal effect shown in Fig. 1A, top trace. The interaction of the competitive antagonist L-NAME with nodal NOS activities was confirmed by overriding the inhibition with excess substrate. L-Arginine was combined with L-NAME in the dialysis inflow at a 10-fold molar excess (5 × 10⁻⁸ mol/min). As shown in Fig. 1A, bottom traces, L-arginine restored vagally mediated bradycardia to values not different from those observed during vehicle administration.

Figure 1B illustrates the results from the entire L-NAME dose response, including the temporal development of the inhibition. The dose response indicates a maximal effect at 5 × 10⁻⁹ mol/min (Fig. 1B, middle) that develops slowly and reaches a maximum between 30 and 45 min of exposure. The dose response was narrow in that no apparent effect was observed at one-tenth the dose (Fig. 1B, left) and no additional effect was observed at three times the dose (Fig. 1B, right). The effect did appear to develop somewhat earlier at the highest dose, suggesting that diffusion to the

Fig. 2. A: heart rate/frequency response during right vagal nerve stimulation at 60 min during the nodal delivery of saline vehicle and the neuronal NOS-1 inhibitor 7-nitroindazole (7 Nitro; 5 × 10⁻⁹ mol/min) alone and combined with L-Arg (50 × 10⁻⁹ mol/min). B: heart rate/frequency response during right vagal nerve stimulations with increasing doses of 7-nitroindazole and with each dose combined with L-Arg (50 × 10⁻⁹ mol/min, 60 min). In an effort to save space, only the 60-min value for L-Arg treatment is shown. Vagal stimulations were performed at 15-min intervals during the 60-min treatments. *P < 0.05, significantly different from control. Values are means ± SE, n = 5.
target may have contributed to the rate of development of the inhibition. Although only the 60-min value is illustrated for the L-NAME/L-arginine combination, the competitive substrate completely prevented the development of the vagolytic effect of L-NAME throughout the hour of exposure at all doses. The reversal by L-arginine indicates that the effect of L-NAME was mediated by NOS inhibition and the failure of L-arginine alone to enhance vagal bradycardia suggested that the endogenous NOS substrate was sufficient under the current experimental conditions.

7-Nitroindazole dose response. The purpose of this experiment was to test whether the NOS activity implicated in vagal transmission in the SA node included the neuronal NOS isoform NOS-1. Control vagal stimulations during vehicle infusion produced consistent step declines in heart rate during vehicle administration throughout the 6-h protocol. The NOS-1-selective inhibitor 7-nitroindazole (5 × 10⁻⁹ mol/min; n = 5) gradually reduced vagal transmission within the SA node by a maximum of 35% (Fig. 2A). An excess of the NOS substrate L-arginine prevented the 7-nitroindazole-mediated reduction in vagal transmission (Fig. 2A) when combined in the dialysis inflow. This reversal by L-arginine provided support for NOS participation in the response to 7-nitroindazole.

Figure 2B illustrates the results of the entire dose response and the temporal development of the effect for comparison with L-NAME above. The maximal effect was evident at 5 × 10⁻⁹ mol/min (Fig. 2B, middle) and appeared to reach that maximum between 15 and 30 min of exposure. The effect was no greater at three times the dose (Fig. 2B, right) and completely absent at one-tenth the dose (Fig. 2B, left). The full inhibition appeared to develop earlier at the highest dose suggesting again that a concentration-dependent factor determines the rate of access to the intracellular target. The combined addition of L-arginine (5 × 10⁻⁸ mol/min) and 7-nitroindazole for 1 h completely prevented the 7-nitroindazole-mediated reduction in vagal bradycardia regardless of the 7-nitroindazole dose employed. To conserve space, only the 1-h values for the L-arginine/7-nitroindazole combination are illustrated as the last bar in each panel. L-Arginine alone did not appear to increase the vagally mediated decline in heart rate compared with that observed during vehicle administration suggesting again that the endogenous substrate available was not limiting during these experimental conditions.

L-arginine versus MEAP. The purpose of this protocol was to test whether enhancing selected components of the NOS-cGMP pathway could reverse or bypass the vagolytic effect of MEAP (n = 5). Figure 3A, top trace, illustrates the vagolytic effect of adding MEAP into the SA node interstitium after 60-min exposure. The dose of MEAP (5 × 10⁻⁹ mol/min) represents the ED₅₀ as
determined in prior studies (20). This dose routinely produces a 65–75% inhibition of vagal bradycardia, as indicated in Fig. 3A. The combined administration of L-arginine and MEAP reversed the vagolytic effect of MEAP (see Fig. 3A, bottom traces). The vagal bradycardia during the administration of L-arginine (5 × 10^{-8} \text{ mol/min}) alone was likewise not different from the vagally mediated bradycardia observed during vehicle administration.

Figure 3B depicts the development of each response during each 1-h portion of the protocol. Figure 3B, left, illustrates the effect of repeated vagal frequency responses during 1 h of vehicle administration. No attrition or enhancement was evident. The addition of MEAP to the dialysis inflow reduced the vagally mediated bradycardia by ∼75% at the first evaluation 15 min later (Fig 3B, middle left). The vagal evaluations throughout the remainder of the hour were not statistically different from the result obtained at 15 min, indicating no significant desensitization of the opioid receptor-mediated vagolytic event. Combining L-arginine and MEAP together did not immediately alter the vagolytic effect of MEAP. A significant reversal of the vagolytic effect was evident only after 45 min of exposure to the combination. The reversal was complete at that point and both the 45- and 60-min values were not different from the initial control vagal responses. After the MEAP/L-arginine combination was washed out, L-arginine was introduced and perfused alone for 60 min. During the administration of L-arginine, the response to vagal stimulation was not different from control and clearly not better than control. These observations suggested that in the absence of inhibition by MEAP or L-NAME, the available supplies of L-arginine are adequate to maintain flux through the NOS cGMP pathway.

Three additional animals were evaluated to test whether the ability of L-arginine to restore vagal function during MEAP administration was consistent with the ability of L-arginine to serve as a NOS substrate. An equal molar dose of the nonsubstrate, D-arginine was substituted for L-arginine in the same protocol to evaluate potential nonspecific actions of the molecule itself. The vagal/heart rate frequency responses at 60 min indicate that D-arginine was unable to modify the vagolytic effect of MEAP (Fig. 4A, top traces) and D-arginine alone had no effect on the vagally mediated decline in heart rate. The complete temporal pattern for D-arginine (Fig. 4B) provided no suggestion of a D-arginine-mediated reversal of MEAP. The sustained vagolytic effect of MEAP during the second hour also provided evidence that the reversal by L-arginine observed in Fig. 3B was not the consequence of a slowly developing tachyphylaxis to MEAP.

**SNAP versus MEAP.** The purpose of this protocol was to test whether NO reverses the vagolytic effect of MEAP consistent with the NOS participation in the reversal. In these experiments, the NO donor SNAP was substituted for L-arginine in the original protocol \((n = 5)\). MEAP produced a typical vagolytic response as illustrated at 60 min in Fig. 5A. The vagal bradycardia was restored to values indistinguishable from those obtained during control when SNAP and MEAP were combined much like the reversal obtained with L-arginine. However, in contrast with L-arginine, SNAP produced a near-complete reversal within 15 min, when the full temporal character of the response was examined (Fig. 5B). SNAP administered alone had no apparent effect on the vagally mediated bradycardia, suggesting that NO production needed for normal vagal transmission is adequate during control conditions.

**cGMP versus MEAP.** The purpose of this protocol was to test whether the ability of NO to reverse the vagolytic effect of MEAP was consistent with the ability of NO to stimulate guanylate cyclase and the accumulation of cGMP \((n = 5)\). In these studies, the cell-permeant cGMP analog 8-bromo-cGMP was substituted for L-arginine in the original protocol. Once again, MEAP produced a clear vagolytic response as illustrated at 60 min in Fig. 6A. Much like L-arginine and SNAP, the cGMP analog completely reversed the vagolytic effect of MEAP when 8-bromo-cGMP and MEAP were combined in the dialysis inflow. The reversal was also evident by 15 min indicating a time course...
more like that observed for SNAP than for L-arginine (Fig. 6B). The cGMP analog did not alter the baseline vagal response when administered alone, suggesting again that flux through this pathway is adequate under control conditions.

Isobutyl methylxanthine versus MEAP. The purpose of this protocol was to test whether the ability of 8-bromo-cGMP to reverse the vagolytic effect of MEAP was consistent with the proposal that cGMP raises cAMP by reducing PDE activity. In these experiments, the PDE inhibitor IBMX was substituted for L-arginine throughout the protocol. The intranodal delivery of MEAP produced a consistent vagolytic response at 60 min, similar to those observed in the earlier experiments (Fig. 7A). These vagolytic effects of MEAP were reversed by the addition of the PDE inhibitor and the heart rate response to vagal stimulation was restored to that observed during vehicle administration. This observation suggested that lowered cAMP contributed to the vagolytic activity of MEAP with an altered PDE activity a potential mechanism. The control responses during vehicle administration and the vagolytic effects of MEAP were each consistent with the previous two experiments, respectively, through the first and second hours of the protocol (Fig. 7B). In contrast to SNAP and 8-bromo-cGMP, the reversal by IBMX developed slowly and became statistically evident between 45 and 60 min in a temporal pattern similar to that observed for the reversal by L-arginine. In the absence of inhibition by MEAP, IBMX had no effect on vagally mediated bradycardia suggesting that the hydrolysis of cyclic nucleotides does not limit vagal transmission during control conditions.

Methacholine versus MEAP. MEAP combined with the direct-acting muscarinic agonist methacholine to test whether the vagolytic effect of MEAP was a direct interaction between MEAP and nodal muscarinic receptors. The nodal delivery of methacholine for 30 min produced a sustained decline in heart rate within the range of heart rates obtained during vagal stimulation (Fig. 8A). After methacholine was washed out, the right vagus was stimulated at 3 Hz, producing a sharp 15-s decline in heart rate (Fig. 8C, left). MEAP was introduced into the dialysis line as a positive control. MEAP did not alter the resting baseline heart rate. After a 5-min exposure, the right vagal stimulus was repeated demonstrating a clear interruption in vagal transmission. The greater change in heart rate during control stimulations also indicated that the effect of methacholine was submaximal in these animals (Fig. 8C, right). Methacholine and MEAP were then combined and infused for a second 30-min interval. The magnitude and temporal pattern of the decline in heart rate were identical to that observed for metha-

Fig. 5. A: heart rate/frequency response to right vagal nerve stimulation at 60 min during the nodal delivery of saline vehicle, the NO donor S-nitroso-N-acetyl-penicillamine (SNAP) alone (5 × 10⁻⁸ mol/min), MEAP (1.5 × 10⁻⁹ mol/min) alone, and MEAP combined with SNAP (5 × 10⁻⁸ mol/min). B: heart rate/frequency response during right vagal nerve stimulation for the entire 60-min time course during saline vehicle, MEAP, SNAP, and MEAP combined with SNAP. Concentrations for each treatment are the same as in Fig. 6A. Vagal stimulations were performed at 15-min intervals. *P < 0.05, significantly different from control. Values are means ± SE, n = 5.
choline alone (Fig. 8A). This observation suggests there was no interaction between MEAP and nodal muscarinic receptors.

**Methacholine versus 7-nitroindazole.** The NOS-1-selective inhibitor 7-nitroindazole (n = 5) was combined with the direct-acting muscarinic agonist methacholine to test whether the vagolytic effect of 7-nitroindazole involved an interaction between 7-nitroindazole and nodal muscarinic receptors. The nodal delivery of methacholine for 30 min produced a sustained decline in heart rate (Fig. 8B) similar to that observed in the prior study. After methacholine was washed out, the right vagus was stimulated at 3 Hz, producing a brief 15-s decline in heart rate. MEAP was introduced into the dialysis line again as a positive control. MEAP did not alter the resting baseline heart rate, but after a 5-min exposure, MEAP produced a clear interruption in vagal transmission (Fig. 8D). This observation suggested the vagolytic effect of 7-nitroindazole observed earlier did not include an interaction between 7-nitroindazole and nodal muscarinic receptors, suggesting that both MEAP and the NOS-1 inhibitor exert their effect proximal to the muscarinic receptors on the pacemaker cells.

**DISCUSSION**

The findings presented in this study are consistent with the hypothesis that MEAP suppressed vagal transmission by interacting with the NOS-cGMP pathway. This interaction was localized within the SA node and was likely prejunctional, occurring in parasympathetic nerve terminals. The comparison of the quantitative and temporal dynamics of vagal inhibition by MEAP and by NOS inhibitors, however, suggested that MEAP and NO may modulate vagal bradycardia by independently converging on a common component, cAMP.

Temporal and quantitative differences between NOS inhibitors and MEAP-mediated vagolysis. Attenuation of vagal bradycardia by the general NOS inhibitor L-NAME and the neuronal NOS inhibitor 7-nitroindazole was quantitatively and temporally dissimilar to MEAP-mediated vagolysis. NOS inhibitors appeared less effective at attenuating vagal bradycardia than MEAP. These agents attenuated vagal bradycardia by 35–50%, whereas MEAP produced a 60–70% inhibition. Temporally, the vagolytic effects of MEAP developed and resolved, respectively, within minutes of ini-
tiating or discontinuing exposure (5). This time frame contrasts with the 45–60 min needed for both NOS inhibitors to take effect. Reversal of the enkephalin-mediated vagolysis by opioid antagonists was also faster compared with reversal by the NOS substrate L-arginine (5, 6, 14, 15, 20). Collectively, these observations suggest that MEAP did not attenuate vagal bradycardia by the direct inhibition of NOS. Rather, observations from experiments employing NO pathway components downstream from NOS suggested that MEAP and NO modulated vagal transmission by convergence on common mediators later in the pathway.

Reversal of MEAP-mediated vagolysis by NOS-cGMP pathway probes. Observations that SNAP, cGMP, and IBMX reversed MEAP-mediated vagolysis were consistent with the hypothesis that opioids and NO could moderate vagal transmission by altering the synthesis and degradation of cyclic nucleotides. The observed effects of NOS pathway intermediates and probes provided support for the sequential participation by nodal guanylate cyclase, PKG, and PDE in the restoration of vagal function during exposure to MEAP. The finding that the PDE inhibitor IBMX reversed the enkephalin-mediated vagolysis suggested that MEAP interrupted vagal transmission by lowering cAMP within the vagal nerve terminals either by suppressing adenylate cyclase or increasing PDE activity. As neuropepti
dors, opioids are widely recognized for their ability to suppress neurotransmitter release through the G/Gi protein-coupled inhibition of adenylate cyclase activity. Because the NOS-cGMP pathway may promote vagal transmission by suppressing PDE activity, the convergence of enkephalin and NO at cAMP provides an attractive explanation of the current findings.

Proposed mechanisms for cholinergic neurotransmission (Fig. 9) include the activation of stimulatory G protein-coupled, cAMP-dependent, second messenger systems (19). In this mechanism, adenylate cyclase activity increases cAMP within presynaptic terminals and activates protein kinase A. The subsequent increase in kinase activity increases neuronal calcium influx and facilitates the vesicular release of acetylcholine. In the current example, acetylcholine binds to muscarinic receptors on nearby pacemaker cells and reduces their rate of spontaneous depolarization leading to a decrease in heart rate (17, 19). Because neuronal opioid receptors interact with inhibitory G proteins that suppress adenyl cyclase (24), the resulting decrease in cAMP would provide a logical explanation of the vagolytic effect of MEAP (24). In contrast, NO promotes vagal transmission by increasing neuronal cAMP indirectly through a reduction in the rate of cyclic nucleotide hydrolysis (17, 18). When neuronal NOS is activated in vagal nerve terminals, the NO

Fig. 7. A: heart rate/frequency response to right vagal nerve stimulation at 60 min during the nodal delivery of saline vehicle, the phosphodiesterase (PDE) inhibitor isobutyl methylxanthine (IBMX) alone (5 × 10^{-8} mol/min), MEAP alone (1.5 × 10^{-9} mol/min), and MEAP combined with IBMX (5 × 10^{-8} mol/min). B: heart rate/frequency response during right vagal nerve stimulation for the entire 60-min time course during saline vehicle, MEAP, IBMX, and MEAP combined with IBMX. Concentrations for each treatment are the same as in A. Vagal stimulations were performed at 15-min intervals. *P < 0.05, significantly different from control. Values are means ± SE, n = 5.
produced activates guanylyl cyclase and increases the concentration of cGMP. Once formed, cGMP activates PKG, which in turn inactivates PDE-3. PDE-3 normally hydrolyzes cAMP to 5’AMP and thus inhibition of PDE-3 leads to the accumulation of cAMP. As stated earlier, an increase in cAMP should facilitate vagal transmission and promote vagal bradycardia (17, 18). The magnitude and temporal character of a PDE-mediated facilitation would however, depend on the existing cyclase activity and ambient concentration of cAMP. As such, the NO pathway may determine the responsiveness of vagal transmission to other neuromodulators that increase or decrease adenylate cyclase activity directly.

Interaction between MEAP and NOS-cGMP pathway is localized prejunctionally in sinoatrial node. The observation that l-NAME, 7-nitroindazole, and MEAP attenuated vagal bradycardia when delivered directly into the SA node suggested that their actions were localized to this region. Prior reports (14) have carefully localized the vagolytic effect of MEAP within the SA node. The interaction between MEAP and selected components of the NOS pathway further suggests that the NOS in question was located within the SA node. This finding does not rule out a similar role for NOS in other parts of the neuroeffector pathway such as the intracardiac parasympathetic ganglion (29). However, the present findings are consistent with reports that have localized neuronal NOS in vagal nerve terminals innervating the mouse and rat SA node and the guinea

Fig. 9. Schematic hypothesis of opioid and NO interactions with neurotransmitter release from parasympathetic nerve terminals in the sinoatrial (SA) node. ACh, acetylcholine; AC, adenylate cyclase; GC, guanylate cyclase; PKA, cAMP-dependent protein kinase; δ2 and M2, δ2-opioid and M2 muscarinic receptors.
pig atria (7, 22). Neither MEAP or the NOS inhibitors appear to interact with cardiac pacemaker cells because all three agents were ineffective when the bradycardia was produced by the intranodal delivery of the direct acting muscarinic agonist methacholine. Similar experiments in the isolated guinea pig atria indicated that NOS inhibitors did not modify the bradycardia produced by the mixed nicotinic/muscarinic agonist carbachol (17). Although that guinea pig model does not distinguish between potential interactions at ganglionic and pacemaker junctions, the data are consistent with the present findings. Thus the opioid and NOS activities in question are likely to be prejunctionally located at the vagal nerve terminals. However, participation by other cells or neural processes within the node have not been systematically ruled out.

The inhibition of vagal transmission by the neuronal NOS isoform-selective inhibitor 7-nitroindazole provided additional circumstantial evidence for the location of the targeted NOS within nodal nerves. The NOS-1 inhibitor 7-nitroindazole appeared less effective than its nonselective analog L-NAME (35% vs. 50%). If this difference is real, another NOS isoform, perhaps endothelial NOS (eNOS) also may have contributed to the overall response. However, immunocytochemical approaches failed to demonstrate eNOS in vagal nerve terminals in the SA node (7) and when eNOS was knocked out, vagal bradycardia was unaltered (7).

Other limitations. Exogenous administration of NO (SNAP) and cGMP reversed the vagolytic effect of MEAP faster than the addition of the NOS substrate L-arginine. This suggested that NOS activity normally restrains the supply of NO despite more than adequate L-arginine concentrations. The reversal pattern by IBMX was slow by comparison suggesting that PKG-mediated inhibition of PDE-3 might be more efficient than that provided by added IBMX. The differences between SNAP, cGMP, and IBMX may also reflect differences in their relative bioavailability in this model system. Although IBMX does slowly reverse the vagolytic effect of MEAP, NO and its target guanylate cyclase may act faster by using more efficient mechanisms than inhibition of PDE.

Physiological significance and evolving hypothesis. Both MEAP and NO appear to function as neuromodulators. Each moderates vagal bradycardia differently though their effects may be integrated by converging on common components of second messenger systems that regulate neurotransmitter release. Both enkephalin and NO probably operate in a slower time domain than the cholinergic transmission they moderate. If, as suggested, MEAP reduces adenylate cyclase activity, then the onset, amplitude, and duration of its influence on vagal transmission will necessarily be determined by the degree of adenylate cyclase inhibition MEAP provides and the relative rate of disposal of existing pools of cAMP by PDE-3. Thus by regulating PDE activity, NO would modulate the responsiveness to enkephalins and other neuromodulators that increase or decrease adenylate cyclase activity. The net effect on transmission would be determined by the integrated sum of influences on synthesis and degradation. The relative ability to moderate synthesis or degradation would depend on the relative catalytic rates of each enzyme. If cyclase activity were high, the influence of PDE would be greater than if the cyclase activity were suppressed. For example, one might expect NO to restore cAMP and vagal transmission slowly during exposure to MEAP because the rate of cAMP production is also reduced. Thus NOS may serve as a tonic background modulator of vagal responsiveness to other perhaps faster, episodic modulators that activate or inhibit adenylate cyclase activity via G protein-coupled mechanisms.

The NOS-cGMP system’s influence may be limited under control conditions because none of the NOS intermediates increased baseline neurotransmission and all were effective only when transmission was first suppressed by enkephalin or NOS inhibitors (7, 17, 18). Within the NOS pathway, NOS may be a point of limitation because bypassing NOS restored vagal transmission faster than adding the NOS substrate L-arginine. Thus chronic changes in a constitutive NOS pathway could be instrumental in determining the responsiveness of the vagus to other neuromodulators. In support of this concept, exercise training-mediated improvements in vagal control of heart rate were well correlated with increased atrial NOS activity (17). Furthermore, pathological states such as hypertension and congestive heart failure are both associated with impaired NO production (16, 26) and impaired vagal control of the heart.

In conclusion, Both MEAP and NO modulate vagal transmission in the canine SA node. Both agents appear to interact but differences in the character of their respective effects suggest independent routes to a common target, perhaps cAMP. Both interactions with vagal transmission are likely prejunctional, presumably mediated within parasympathetic nerve terminals.

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