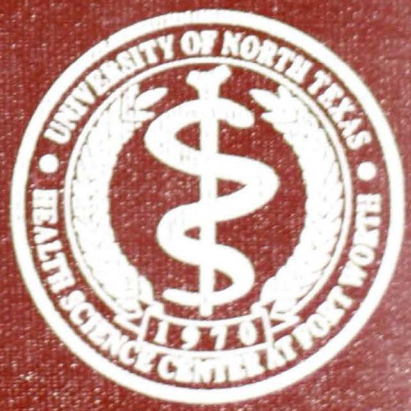

UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

Farias III, Martin, Opioid and Nitric Oxide Interaction in the Control of Heart Rate. Doctor of Philosophy (Biomedical Sciences), December 2002, 130 pp, 2 tables, 30 figures.

Understanding of the role endogenous opioids play as modulators of parasympathetic function has increased. The endogenous opioid, methionine-enkephalin arginine phenylalanine (MEAP) attenuates vagal control of heart rate when delivered by microdialysis directly in the canine sinoatrial node. This effect was mimicked by the δ-2 agonist, deltorphin-II indicating involvement by a δ-opioid receptor. The nodal delivery of the δ-antagonist naltrindole abolished the effect of deltorphin-II, further supporting the delta character of the receptor. Although the findings suggested that the opioid receptor mediating vagolysis was delta in character, the exact subtype of δ-receptor remained in question.

Selective agonists and antagonists for δ-1 and δ-2-opioid receptors were employed to determine which subtype of δ-receptor mediated MEAP vagolysis. In these experiments, vagolysis produced by the nodal delivery of MEAP was unaltered by the highly selective δ-1 antagonist BNTX but abolished by the δ-2 antagonist, naltriben. Nodal delivery of deltorphin-II attenuated vagal bradycardia similar to MEAP while δ-1 agonists, DPDPE and TAN-67 failed to interrupt vagal bradycardia. TAN-67 actually improved vagal transmission and this effect was reversed by BNTX. These data indicate that δ-2-opioid receptors in the sinoatrial node are vagolytic and support the presence of vagotonic δ-1-opioid receptors in the same location.

Nitric Oxide/ Opioid Interaction

The hypothesis that intranodal nitric oxide synthase (NOS) modulates vagal transmission and that MEAP attenuates vagal bradycardia via the interruption of the NOS-cGMP pathway was tested. The general (L-NAME) and neuronal (7-nitroindazole) NOS inhibitors each attenuated vagal bradycardia and both effects were reversed by adding excess of the NOS substrate, L-arginine. These findings suggested that nNOS was a necessary component of vagal bradycardia in the canine sinoatrial node.

Various probes of the NOS-cGMP pathway (L-arginine, SNAP, cGMP, and IBMX) were employed to determine if MEAP interrupted this pathway to produce vagolysis. The delivery of MEAP into the sinoatrial node for sixty minutes exerted a consistent vagolytic effect during vagal stimulations. When MEAP was combined with NOS pathway components, the vagolytic effect was reversed after 15-45 minutes of treatment. These findings suggested that MEAP exerted its effect by interacting with the NOS-cGMP system. The site of convergence maybe cAMP since the phosphodiesterase inhibitor, IBMX (by allowing the accumulation of cAMP) reversed the vagolytic effect of MEAP.

To rule out a postjunctional effect, MEAP and the NOS inhibitors were combined with the direct acting muscarinic agonist, methacholine. The bradycardia produced by methacholine was unaltered by MEAP or nNOS inhibitors. This suggested that the effect of NOS inhibitors and MEAP was prejunctional. In summary, the cumulative findings suggest that MEAP, by activating δ-2-opioid receptors, attenuated vagal bradycardia prejunctionally, through modulating the cAMP component of the NOS-cGMP pathway in the canine sinoatrial node.

OPIOID AND NITRIC OXIDE INTERACTION IN

THE CONTROL OF HEART RATE

Martin Farias III, M.S.

APPROVED:

James L Caffrey
Major Professor

H. Fred Downey
Committee Member

Patricia A. Gariss
Committee Member

Michael W. Martini
Committee Member

Holm P. Malm
Committee Member

[signature]
University Member

Patricia A. Gariss
Chair, Department of Integrative Physiology

[signature]
Dean, Graduate School of Biomedical Sciences

OPIOID AND NITRIC OXIDE INTERACTION IN THE CONTROL OF HEART RATE

DISSERTATION

Presented to the Graduate Council of the Graduate School of

Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

Martin Farias III

Fort Worth, Texas

December 2002

ACKNOWLEDGEMENTS

This project was supported by a National Research Service Award from the National Institutes of Health and by a Texas ATP grant at the University of North Texas Health Science Center.

This endeavor was not possible without the support, guidance, patience, and expertise of my mentor, Dr. James Caffrey. I have learned a great deal about the effort and heart it takes to do **Good Science.** I am truly indebted to Dr. Caffrey for helping me fulfill my potential and mature as an individual. I would also like to thank Keith Jackson, Darice Yoshishige, and Amber Stanfill for their help with these studies. I also express my thanks to members of S.A.L.S.A. (the Society for the Advancement of Latino Scholars in Academia) for their support and friendship. Members and staff of the Graduate School of Biomedical Sciences and the Department of Integrative Physiology were very much a part of my experiences during my studies. I learned a great deal from every individual. To them I offer my sincere gratitude. To my parents, Mary Lou Farias and Martin Farias who never gave up on me and always steered me towards a better future, thank you. To my grandparents, Daniel and Frances Ramirez for their unconditional love and support, thank you. To my beautiful wife, Valerie Farias, I love you and thank you for your patience, care and understanding and most of all for giving life to my beautiful daughter, Gianna Marcela Farias, whom now is my life.

Original Articles

Farias M, Jackson K,. Stanfill A, Caffrey JL, Local opiate receptors in the sinoatrial node moderate vagal bradycardia. *Auto Neuro* 87:9-15,2001.

Jackson K, Farias M, Caffrey JL, Cardiac microdialysis is a powerful tool. *Cardiovas Res* 46(3): 376-379, 2001.

Jackson K, Farias M, Stanfill AA, Caffrey JL. Transient Ischemia raises enkephalin in the sinoatrial node and improves vagal bradycardia. *Auto Neuro* 94: 84-92,2001.

Jackson K, Farias M, Stanfill AA, Caffrey JL. Delta opioid receptors inhibit vagal bradycardia in the sinoatrial node. *Cardiovas Pharmacol Ther* 6 (4): 385-393, 2001.

Stanfill AA, Jackson K, Farias M, Caffrey JL. Leucine-enkephalin and the local sympathetic control of heart rate in the SA Node. Submitted: *Exp Biol Med*, 2002.

Farias M, Jackson K, Yoshishige D, Caffrey JL. Cardiac enkephalins interrupt vagal bradycardia via δ-2 opioid receptors in the sinoatrial node. 2002 In Press: *Am J Physiol (Heart Circ Physiol).*

Abstracts

Farias M, Jackson K , Stanfill AA, Caffrey JL . Prejunctional opiate receptors in the SA node modulate vagal bradycardia. *FASEB J* 13 (5) part I: A453, 1999.

Jackson K, Stanfill AA, Farias M , Caffrey JL. Delta opiate receptors block the vagolytic effect of MEAP in the sa node. *FASEB J* 13 (5) part I: A453, 1999.

Jackson K, , Stanfill A, Farias M, Caffrey JL. MEAP alters sinoatrial node response during ischemia. *INRC J*, 1999.

Jackson K, Farias M, Stanfill AA, Caffrey JL. Delta receptor modulates vagal control of heart rate at the sinoatrial node. *FASEB journal* 14 (4)A378, 1999.

Farias M, Jackson K , Stanfill AA, Caffrey JL. Super Dalda enhances adrenergic responses in the dog. *INRC J*, 2000.

Jackson K , Farias M, Stanfill AA, Caffrey JL. Pharmacological profiles indicate vagolytic opiate receptors in the sa node are delta specific. *INRC J*, 2000.

Farias M, Jackson K , Stanfill AA, Caffrey JL. DPDPE, a delta-1 agonist , does not inhibit vagal bradyardia in the dog. *FASEB J*, 2001.

Farias M, Jackson K , Johnson M, Caffrey JL. Nitric oxide synthase in the sinoatrial node of the dog participates in vagal function. *FASEB J* 16 A105 (126.5) 2002.

Stanfill AA, Jackson K, Farias M, Caffrey JL. Leucine-enkephalin and the local sympathetic control of heart rate in the sa node. Research Appreciation Day abstract book, UNTHSC-FW, April 5, 2002

Farias M, Jackson K , Caffrey JL. Tan-67 improves rather than disrupts vagal bradycardia. *INRC J*, Asilomar, CA , July 9 ,2002.

Jackson K, Farias M, Yoshishige D, Caffrey JL. Bimodal opioid effects on vagal bradycardia. *INRC J*, Asilomar, CA , July 9 ,2002.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

Figure

CHAPTER II.

Figure

CHAPTER III

Figure

LIST OF TABLES

CHAPTER 1

INTRODUCTION

The investigations discussed in this dissertation were performed to examine the paracrine physiology of the canine sinoatrial node. A novel microdialysis tool was implemented to specifically investigate opioid and nitric oxide interactions with respect to parasympathetic nerve action on the sinoatrial node. Recent investigations of nodal physiology have demonstrated the following.

1. The endogenous enkephalin MEAP is a vagolytic agent (6,7,18).
2. Reduced blood flow raises endogenous levels of MEAP in the sinoatrial node (28).
3. Endogenous MEAP is vagotonic during reduced blood flow (28).
4. MEAP mediated vagolysis employs δ-opioid receptors in the sinoatrial node (27).

The subtype of δ-opioid receptor mediating the vagolytic effect of MEAP was unclear. Since the δ-2 agonist, deltorphin-II mimicked the MEAP mediated vagolysis, the δ-2 receptors are likely candidates(27). The NOS-cGMP pathway is thought to be a requisite component of vagal bradycardia and opioids have been shown to inhibit vagal bradycardia (8,9,12,13,16,22,23,45,46). The activation of these δ-2 receptors by MEAP could be suppressing parasympathetic function by interrupting the NOS-cGMP pathway in vagal nerve terminals.

The objectives of this dissertation were four fold: first, to delineate which subtype of δ-opioid receptor in the sinoatrial node was responsible for MEAP mediated vagolysis; second to show that neuronal NOS in the sinoatrial node was necessary for normal vagal bradycardia ; third, to test whether MEAP interrupted the NOS-cGMP pathway in the sinoatrial node to mediate its vagolytic effect, and fourth, to show that the effect of MEAP and NOS were prejunctional in the sinoatrial node. The studies that followed employed a variety of opioid agonists and antagonists and various components of the NOS-cGMP pathway to accomplish the objectives described above.

REVIEW OF THE RELATED LITERATURE

Control of heart rate

The cardiac autonomic nervous system consists of two major limbs: the sympathetic nervous system increases heart rate and contractile force while the parasympathetic nervous system decreases both these cardiovascular parameters. Autonomic nerve terminals converging on pacemaker cells of the sinoatrial node are necessary for this modulation of heart rate (24). When activated, sympathetic nerves release norepinephrine which binds to and activates postjunctional β-1 adrenergic receptors. This action induces a cascade of intracellular events mediated by the cyclic adenosine monophosphate (cAMP) second messenger system. One of these events is an increase in Ca^{2+} influx into pacemaker cells which increases their spontaneous rhythm. This translates into an increase in heart rate (24). The exact opposite happens when acetylcholine is released from cholinergic nerve terminals. Acetylcholine activates muscarinic receptors on

pacemaker cells and decreases heart rate by suppressing the cAMP second messenger system and reducing the influx of Ca^{2+} into these cells (24). Though the sympathetic and parasympathetic innervation of the sinoatrial node predominates, little is known about the paracrine influences that fine tune the local environment in which they operate. Opioid and nitric oxide systems are gaining recognition as local modulators of cardiac innervation and ultimately, autonomic balance.

Endogenous Opioids: Discovery, Processing and Endogenous Opioid Receptors

The term opioids refers to compounds having pharmacological properties similar to opium. Opium is a mixture of pharmacologically active agents extracted from the opium poppy, *Papaver Somniferum.* Opium has been used for its medicinal analgesic and antidiarrheal properties for centuries (20,36).

The alkaloid, morphine is the active agent in opium (20,33,36). The addictive properties and potential for abuse have hampered the free use of morphine for medicinal purposes. (20,33,36). As a result pharmaceutical chemists for the past century have tried to separate the medicinal and addictive properties of this compound. Unfortunately, these attempts have been unsuccessful.

Endogenous opioids are agents produced by the body that have properties similar to opioid alkaloids derived from the opium poppy (36). Various groups have brought forth evidence supporting the existence of these compounds. One of the most salient findings

was provided by Kosterlitz et al (33) when they observed that brain extracts could block the release of acetylcholine in guinea pig ileum, a standard opioid bio-assay. This effect was reversed by the opiate antagonist naloxone, which suggested that this substance was an endogenous agonist for the opiate receptor. The active ingredients were isolated and sequenced as two similar pentapeptides; methionine enkephalin (Tyr-Gly-Gly-Phe-Met) and leucine enkephalin (Tyr-Gly-Gly-Phe-Leu) (26). These peptides were immediately identified in other tissues including the heart (26, 54).

Processing

Proenkephalin, the precursor for the pentapeptides described above, was isolated from bovine adrenal cortex and cloned from both bovine and human tissues (25, 39) Proenkephalin is also concentrated in the heart (54). This precursor contains four different opioid sequences including: four copies of methionine enkephalin and one each of leucine enkephalin, methionine-enkephalin-arg-phe, and methionine-enkephalin-arg-gly-leu (figure 1). These enkephalins are liberated by the hydrolysis at pairs of basic amino acids surrounding each sequence. When liberated, they are biologically active and their half-life is dictated by circulating proteases and peptidases (54).

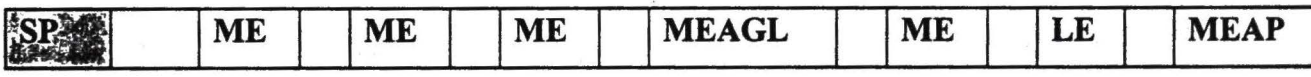


Figure 1. Schematic Representation of Proenkephalin (31 K)

Endogenous Opioid Receptors

There are three classes of endogenous opioid receptors generally labeled μ, κ, and δ. The characterization of these receptors was determined through a variety of behavioral, neurophysiological and pharmacological investigations (20,36). Distinct agonist and antagonist profiles led to the proposal of these receptor classes. Opioid receptors regulate a variety of physiological systems primarily by altering neurotransmitter release (20,36). The receptor classes are complicated further by similar evidence for the existence of subtypes of these receptors such as δ-1 and δ-2 subtypes (17,29,30,42,47,48).

Recent antinociceptive and receptor binding studies with δ-opioid agonists and antagonists have provided functional evidence for distinct δ receptor subtypes (11,17,29,30,37,42,47,48,50,55). Despite the isolation of a single δ receptor protein sequence, evidence persists for separate subtype specific biological actions of δ-1 and δ-2 receptors. For example, Schultz et al (43) demonstrated that pretreatment with the selective δ-1 agonist, TAN-67, reduced infarct size in the ischemic rat heart. The cardioprotection was prevented by a selective δ-1 antagonist and unaltered by a selective δ-2 antagonist. In contrast, Jackson et al (27) showed that the δ-2 agonist, deltorphin-II attenuated vagal bradycardia in the dog. The circumstances that determine which subtype is manifest remains poorly defined.

Opioid Control of Cardiac Parasympathetic Function

Various groups have reported that endogenous opioids inhibited parasympathetic function (5,6,7,27,28,38,41,53). One endogenous opioid in particular, the cardiac enkephalin, methionine-enkephalin-arginine-phenylalanine (MEAP) exhibited significant vagolytic activity in the dog. MEAP attenuated vagally mediated bradycardia by more than seventy percent when infused systemically. MEAP was more potent than its close relative, methionine enkephalin. The high affinity but nonselective opioid antagonist, diprenorphine completely reversed the peptide effect and restored vagal bradycardia, indicating opiate receptors were involved (6,7).

These findings suggested that the vagolytic effect was peripheral in character though the location and specific receptor remained unclear. Likely targets included the intracardiac parasympathetic ganglion and prejunctional vagal nerve terminals innervating the sinoatrial (SA) node. A site in the sinoatrial node was identified since the delivery of MEAP directly into this region by microdialysis attenuated vagally mediated bradycardia to the same extent as the systemic infusion of the peptide (18). The nodal location was confirmed by the fact that the vagolytic effect of systemically administered MEAP was blocked by the local introduction of diprenorphine into the nodal interstitium. Collectively, these findings indicated that MEAP modulated vagal control of heart rate by acting on opioid receptors located in the sinoatrial node (18).

The nature of the receptor however remained in question. Subsequent studies have indicated that the vagolytic effect of MEAP was mediated by δ-opioid receptors. In an extended series of dose responses with specific opioid agonists and antagonists, Jackson et al (27) established a clear receptor profile. Nodal delivery of increasing concentrations of MEAP blocked vagally mediated bradycardia. Deltorphin II, a selective δ-2 opioid receptor agonist, attenuated vagal bradycardia similar to MEAP but more profoundly. The delta selective antagonist, naltrindole completely reversed the vagolytic actions of both MEAP and deltorphin II. In the same experiments, and agonists had no effect on vagally mediated bradycardia and their respective antagonists were ineffective versus MEAP (27). Together, these data strongly indicated that δ-opioid receptors within the sinoatrial node were responsible for the vagolytic effect of MEAP.

MEAP in the Sinoatrial Node

The actions of endogenous opioids in the cardiovascular system are not well characterized. However, MEAP is found highly concentrated in the heart (28,54). The precursor, proenkephalin is also in abundance in the myocardium (25). More interestingly, MEAP can be recovered from the sinoatrial node. Jackson et al (28) using radioimunnoassay found that MEAP concentrations increased during a preconditioning like protocol . However, the local production of MEAP exerted a vagotonic rather than a vagolytic effect. This observation suggested that endogenous and exogenous peptides exerted different responses during normal and reduced blood flow. The nodal location of

MEAP suggests that it may serve as an intricate paracrine regulator of parasympathetic function.

Preconditioning Phenomenon and Endogenous Opioids

Brief periods of coronary artery occlusion followed by reperfusion are known to protect the heart from the damage incurred by a prolonged occlusion. This phenomenon is known as preconditioning. In ischemic rat hearts, infarct size as a percent of area at risk (IS/AAR) was greatly reduced in hearts treated with a preconditioning protocol (4, 44). Recent studies have shown that there are a variety of mediators for this phenomenon. Of particular interest is the observation that opioids can confer cardioprotection during ischemic events. Schultz et al showed that morphine reduced the IS/AAR in rats challenged with ischemia. The protective effect was reversed by the nonselective opiate antagonist naloxone, and the δ-selective antagonist, naltrindole (44). The preconditioning effect was not only mediated by an opioid receptor but also that receptor was δ in nature.

Since there is evidence for the existence of two pharmacologically distinct δ-opioid receptors (δ-1 and 2), Schultz et al demonstrated that a δ-1 agonist (TAN-67) and not a δ-2 agonist reduced infarct size in ischemic rat hearts. The effect of TAN-67 was abolished by pretreatment with the δ-1-opioid antagonist, 7-benzyllidenenaltexone (BNTX). BNTX treatment also reversed the cardioprotection conferred by brief cycles of occlusions and reperfusions (43). These findings provide evidence that δ-1 opioid receptors were

cardioprotective and that the ischemic rat heart contained an opioid able to confer this protection. The opioid may be MEAP since Jackson et al (27) found that occlusion and reperfusion of the SA node artery released MEAP. This resulting increase in vagal transmission may reduce oxygen consumption locally and thus reduce ischemic damage.

Bimodal Opioid Effect

The fact that exogenously administered MEAP produced a vagolytic response and endogenously released MEAP was vagotonic was puzzling. However, work done by Crane et al (14) provide an interesting hypothesis for these observations. Very low opioid concentrations exert stimulatory effects that are opposite to the inhibitory effects produced by traditional concentrations of opioids (14).

The proposed mechanism though complex in nature is consistent with current observations. On the biochemical level certain lipid chaperones (e.g. GM1) are thought to translocate stimulatory G (Gs) proteins to opioid receptor sites during treatment with low concentrations of opioids. This action produces a shift from an inhibitory to an excitatory dose response curve (14). A similar shift from inhibitory to excitatory actions during ischemia could explain the disparate effects of endogenous and exogenous MEAP. Small amounts of MEAP could activate excitatory δ-opioid receptors present in the sinoatrial node. This could confer cardiac protection by increasing vagal tone to decrease metabolic demand during reduced oxygen delivery.

Nitric Oxide

During the last decade numerous reports have described the actions of nitric oxide (NO) (8,9,21). Nitric oxide is a labile product from the conversion of L-arginine to L-citrulline by the enzyme, nitric oxide synthase (NOS). The vascular properties of NO were elucidated by Furchgott et al when they determined that the physiological and physicochemical properties of endothelium derived relaxing factor were similar to NO (8,9,21). NO is produced in various tissues by three principal isoforms of NOS. The main actions of NO include smooth muscle relaxation, inhibition of platelet aggregation and monocyte adherence (8,9,21).

Isoforms of NOS

The three isoforms of NOS were first identified in brain, macrophages, and endothelial cells (8,9,21). These isoforms include neuronal (found in brain and peripheral nerves, or NOS-1), inducible (found in a variety of cells, or NOS-2), and endothelial (found in endothelial cells of arterioles, or NOS-III). Subsequent studies have since localized NOS-1 and III to a wide variety of other tissues such as skeletal muscle, cardiac muscle, and autonomic nerves (21).

These three isoforms have many actions (4,21). NOS-III has been the most studied isoform of NOS. NOS-III relaxes smooth muscle and inhibits platelet aggregation. NOS-I can regulate the release of neurotransmitters from both cholinergic and adrenergic nerve terminals and can also induce metabolic dilation of skeletal muscle during exercise.

NOS-II may mediate certain pathologies such as heart failure and is also a mediator of preconditioning in the heart (4,21).

Intracellular NO Targets

NO has many potential intracellular targets (21,23). The most studied of these targets is soluble guanylyl cyclase (GC) . NO activates GC by binding to its heme moiety and inducing a conformational change in this protein. Once activated, GC synthesizes cyclic guanosine monophosphate (cGMP). cGMP activates protein kinase G (PKG), which in turn modulates ion channels and enzymes. Of particular interest, cGMP inhibits the activity of phosphodiesterases, which degrades cyclic adenosine monophosphate (cAMP) (21, 23). Other potential NO targets include membrane thiols, ribonucleotidases and NADPH oxidases (21).

Role of Neuronal NOS in Parasympathetic function

Recently neuronal NOS was identified in parasympathetic nerve terminals innervating the region around the sinoatrial node of guinea pig and rat heart (32,51). Neuronal NOS in this area has been hypothesized to be an integral part of normal vagal bradycardia. Herring et al (22) have shown that treatment of guinea pig atrial preparations with nNOS inhibitors attenuated vagally mediated bradycardia which was subsequently reversed by excess of the NOS substrate, L-arginine. These findings suggested that NOS-I and more specifically NO were constitutive components of normal vagal transmission.

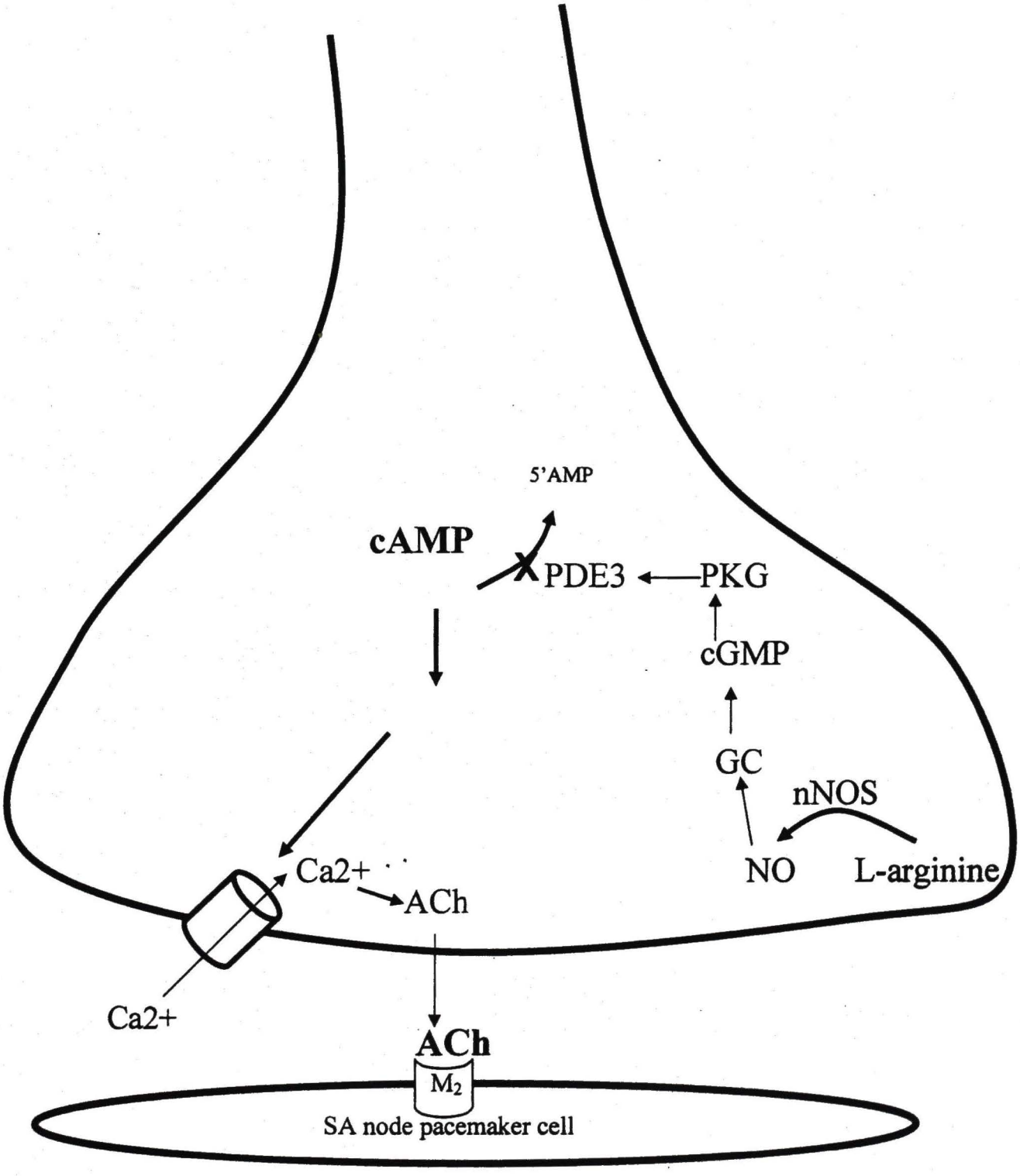
The proposed mechanism of vagal transmission involves the NOS-cGMP pathway. Increased NO flux during vagal nerve stimulation activates GC and increases the formation of cGMP (figure 2). cGMP activates PKG, which phosphorylates and inhibits phosphodiesterase 3 (PDE-3), causing cAMP to accumulate. This accumulation of cAMP activates PKA, which then phosphorylates Ca^{2+} channels and increases Ca^{2+} influx into parasympathetic nerve terminals. The rising intracellular Ca^{2+} facilitates the vesicular release of acetylcholine and initiates the subsequent bradycardia (22,23).

Endogenous Opioid and nNOS Interaction in the Control of Heart Rate

There is evidence that opioids can exert control over the NOS-cGMP pathway (1,2,15,34,49,52). Bhargvana et al (1,2) demonstrated that δ-opioid agonists exhibited an inhibitory effect on the synthesis of NO and cGMP in rat spinal cord preparations. Endogenous opioids may also exert an inhibitory effect on the NOS-cGMP pathway in the SA node. However, there are no current studies in the literature to support this hypothesis.

NOS and vagal bradycardia

Figure 2



Summary

The endogenous opioid, MEAP, is well positioned as a modulator of parasympathetic function. MEAP is abundant in the heart, localized in the sinoatrial node where access to vagal nerve terminals facilitates opioid modulation of vagal bradycardia (6, 28, 54). Nitric oxide is an integral part of parasympathetic transmission. The NO pathway is localized within parasympathetic nerve terminals in the sinoatrial node area and facilitates vagal bradycardia (23,32). Thus, MEAP may interact with NO to modulate vagal bradycardia.

MEAP exerted its vagolytic actions through δ-opioid receptors in the sinoatrial node (27). Preliminary data also suggested that MEAP might exert its vagolytic action by suppressing the NO-cGMP pathway. These findings led us to propose the following hypothesis: **MEAP acting on δ-2 opioid receptors located pre-junctionally in the sinoatrial node suppresses vagal bradycardia by interrupting the nNOS-cGMP pathway.**

SPECIFIC AIMS

Specific Aim 1 tested whether the δ-2 opioid receptors in the sinoatrial node were responsible for the vagolytic effect of MEAP. Previous studies showed that MEAP acted on δ-opioid receptors to inhibit vagal bradycardia and suggested that a δ-2-receptor subtype may have been responsible (27). Assorted agonists and antagonists selective for δ-receptor subtypes were used to accomplish this aim.

Specific Aim 2 Tested whether NOS activity in the sinoatrial node participated in vagal bradycardia in the dog. Herring et al (21) showed that general and neuronal NOS inhibitors attenuated vagal bradycardia in isolated guinea pig atria. Their findings suggested that nNOS was a requisite part of vagally mediated bradycardia but did not specifically identify the site as intranodal. This study determined the location of this effect. This aim was accomplished by combining nodal microdialysis with general and neuronal NOS inhibitors delivered into the SA node during vagal stimulations.

Specific Aim 3 Tested whether nodal MEAP suppressed vagal bradycardia by interrupting the NOS-cGMP pathway in the sinoatrial node. Several reports indicated that opioids exerted inhibitory actions on the NOS-cGMP pathway in other tissues. Since NOS appears to facilitate vagal bradycardia and MEAP is vagolytic, MEAP logically might inhibit vagal bradycardia by interrupting the NO pathway. MEAP and various components of the NOS-cGMP pathway were combined to accomplish this aim.

Specific Aim 4 Tested whether MEAP and NOS inhibitors interrupted vagally mediated bradycardia by interaction with muscarinic receptors within the node to attenuate vagal bradycardia. Herring et al. (22) showed that general and neuronal NOS inhibitors could not overcome the bradycardia induced by the mixed cholinergic agonist carbamylcholine, suggesting an interaction proximal to the pacemaker cells. Neuronal NOS has also been located in cardiac parasympathetic nerve terminals in the rat sinoatrial node area (32). Systemic MEAP was unable to overcome the actions of the direct acting muscarinic agonist methacholine, suggesting again a pre-junctional mechanism for inhibition of vagal bradycardia (6).

Significance

The paracrine environment of the sinoatrial node during normal and pathological states is not well characterized. This study provides insights pertaining to the functions of endogenous opioids and nitric oxide in the sinoatrial node environment and their effect on parasympathetic function. Vagal control of the heart is important to survival (3,31). The rapid hydrolysis of acetylcholine by local acetylcholinesterases allows the vagus to efficiently regulate heart rate on a beat to beat basis. In congestive heart failure and myocardial infarction, there is an increase in endogenous opioids, enhanced sympathetic tone, and an impairment of the NO system (4,19,21,35). These conditions depress vagal transmission and allow the sympathetic nervous system to exert control of the heart (4,21). This scenario increases oxygen consumption and reduces efficiency, which are

associated with an increased probability of arrhythmia, myocardial infarct, and sudden cardiac death (40). Studies have shown that patients who regain vagal control are more likely to survive these cardiac events (3,31). The findings in this study provide some insight into potential mechanisms of vagal dysfunction and may lead to treatments that restore vagal transmission during these events.

REFERENCES

1. **Bhargava HN, and Cao YJ**. Effect of chronic administration of [D-Pen2, D-Pen5] enkephalin on the activity of nitric oxide synthase in brain regions and spinal cord of mice. *Peptides* 19: 113-7, 1998.

2. **Bhargava HN, and Cao YJ**. Effect of chronic administration of morphine, U-50, 488H and [D-Pen2, D-Pen5] enkephalin on the concentration of cGMP in brain regions and spinal cord of mice. *Peptides* 18: 1629-1634, 1997.

3. **Billman G E, S chwartz P J, a nd S tone H** L. The effects of daily exercise on the susceptibility to sudden cardiac death. *Circulation* 69: 1182-1189, 1984.

4. **Bolli R**. The late phase of preconditioning. *Circ. Res*. 87: 972-983, 2000.

5. **Caffrey JL, Wooldridge CB, and Gaugl JF**. The interaction of endogenous opiates with autonomic circulatory control in the dog. *Circ. Shock*, 17: 233-242, 1985.

6. **Caffrey JL, Mateo Z, Napier LD, Gaugl JF, and Barron BA**. 1 995. I ntrinsic cardiac enkephalins inhibit vagal bradycardia in the dog. *Am J Physiol (Heart Circ Physiol* 37) 268: H848-H855,1995.

7. **Caffrey JL.** Enkephalin inhibits vagal control of heart, contractile force, and coronary blood flow in the dog in vivo. *J Auto Nerv Syst* 2318: 1-8, 1999.

8. **Chowdhary S, Vaile JC, Fletcher J, Ross HF, Coote JH, and Townend JN.** Nitric oxide and cardiac autonomic control in humans. *Hypertension* 36:264-269, 2000.

9. **Chowdhary S, and Townend JN.** Role of nitric oxide in the regulation of cardiovascular autonomic control. *Clin. Sci.* 97:5-17, 1999.

10. **Chien S, Oeltgen PR, Diana JN, and Salley RK.** Extension of survival time in multi organ block preparation with a delta opioid DADLE. *J Thorac Cardiovascular Surg* 107: 964-967, 1994.

11. **Clarke GD, and Dondio GM.** Recent advances in the development of a non-peptide delta opioid analgesic. *Pharmacol Rev Commun* 9: 49-60, 1997.

12. **Conlon K, and Kidd C.** Neuronal Nitric oxide facilitates vagal chronotropic and dromotropic actions on the heart. *J Auton Nerv Sys* 75:136-146, 1999.

13. **Conlon K, Collins T, and Kidd C.** The role of nitric oxide in the control by the vagal nerves of the heart of the ferret. *Exp. Physiol* 83: 469-480, 1998.

14. **Crain SM, and Shen KF**. Modulation of opioid analgesia, tolerance and dependence by Gs-coupled, GM`1 ganglioside-regulated opioid receptor functions. (Review). *TIPS* 19: 358-365, 1998.

15. **Deliconstantinos G, Villiotou V, and Stavrides JC**. Met- enkephalin receptor mediated increase of membrane fluidity modulates nitric oxide and cGMP production in rat brain synaptosomes. *Neurochem. Res*. 20: 217-224, 1995.

16. **Elvan A, Rubart M, and Zipes DP**. NO modulates autonomic effects on sinus discharge rate and AV nodal conduction in open chest dogs. *Am. J. Physiol.*272 (*Heart Circ. Physiol.* 41): H263-H271, 1997.

17. **Erspamer V, Melchiorri P, Flaconieri-Erspamer G, Negri L, and Corsi R.** Deltorphins: a family of naturally occurring peptides with a high affinity and selectivity for delta opioid binding sites. *Proc Natl Acad Sci USA* 86: 5188-92, 1989.

18. **Farias M, Jackson K, Stanfill A, and Caffrey JL.** Local opiate receptors in the sinoatrial node moderate vagal bradycardia. *Auto Neurosci (Basic and Clin)* 87: 9-15, 2001.

19. **Fontana F, Bernardi P, Pich EM, Capelli M, Bortoluzzi L, Spampinato S, and Canossa M.** Relationship between plasma atrial natriuretic factor and opioid levels in

healthy subjects and in patients with acute congestive heart failure. *Eur Heart J* 14: 219-225, 1993.

20. **Goldstein A, and Naidu A.** Multiple opioid receptors: ligand selectivity profiles and binding signatures. *Mol Pharmacol* 36: 265-72, 1989.

21. **Hare JM, and Colucci WS.** Role of nitric oxide in the regulation of myocardial function. *Prog. Cardiovasc. Dis.* 38 (2): 155-66,1995.

22. **Herring N, Golding S, and Paterson DJ.** Pre-synaptic NO-cGMP pathway modulates vagal control of heart rate in isolated adult guinea pig atria. *J. Mol. Cell Cardiol.* 32: 1795-1804, 2000.

23. **Herring N, Danson EJF, and Paterson DJ.** Cholinergic control of heart rate by nitric oxide is site specific. *NIPS* :202-206,2002.

24. **Horackova M, and Armour JA.** Role of peripheral autonomic neurones in maintaining adequate cardiac function. *Cardiovasc Res* 30 : 326-335, 1995.

25. **Howells R, Kilpatrick D, Bailey C, Noe M, and Udenfriend S.** Proenkephalin mRNA in rat heart. *Proc Natl Acad Sci* 83: 1960-1963, 1986.

26. **Hughes J, Smith T, Kosterlitz H, Fothergill L, Morgan B, and Morris H.** Identification of 2 related pentapeptides from the brain with potent opiate agonist activity. *Nature*, 258: 577-579, 1975.

27. **Jackson KE, Farias M, Stanfill A, and Caffrey JL**. Delta opioid receptors inhibit vagal bradycardia in the sinoatrial node. *J Cardiovasc Pharmacol Therapeut* 6(4): 385-393, 2001.

28. **Jackson KE, Farias M, Stanfill, and Caffrey JL.** Transient arterial occlusion raises enkephalin in the sinoatrial node and improves vagal bradycardia. *Auto Neurosci* (*Basic and Clin*) 94: 84-92, 2001.

29. **Jiang Q, Takemori AE, Sultana M, Portoghese PS, and Bowen WD**. Differential antagonism of delta opioid antinociception by [D-ala2, Leu5, Cys6]-enkephalin and 5'- naltrindole isothiocyanate (5'-NTII): evidence for delta receptor subtypes. *J Pharmacol Exp Ther* 257: 1069-75, 1991.

30. **Kamei J, Saitoh A, Ohsawa M, Suzuki T, Misawa M, Nagase H, and Kasuya Y**. Antinociceptive effects of the selective non-peptidic delta opioid receptor agonist TAN-67 in diabetic mice. *Eur J Pharmacol* 276: 131-135, 1995.

31. **Kleiger RE, Miller LP, and Bigger JT**. Decreased heart rate variability and its association with increased mortality after acute myocardial infarction. *Am J Cardiol* 59: 256-262, 1987.

32. **Klimaschewsky L, Kummer W, Mayer B, Courand JY, Preissler U, Philippin B, and Heym C.** Nitric oxide synthase in cardiac nerve fibers and neurones of rat and guinea pig heart. *Circ. Res.* 71: 1533-1537, 1992.

33. **Kosterlitz H, and Waterfield A.** In vitro model in the study of structure activity relationships of narcotic analgesics. *Nature* 258: 577-579, 1975

34. **Lenard L, Halmai V, and Bartho L.** Morphine contracts the Guinea pig ileal circular muscle by interfering with a nitric oxide mediated tonic inhibition. *Digestion* 60: 562-6, 1999.

35. **Llobel F, and Laorden ML.** Effects of mu-, delta- and kappa- opioid antagonists in atrial preparations from nonfailing and failing human hearts. *Gen Pharmacol* 28: 371-374, 1997.

36. **Martin WR.** Pharmacology of opioids. *Pharmacol Rev* 35: 285-323, 1984.

37. **Mattia A, Vanderah T, Mosberg HI, and Porreca F.** Lack of antinociceptive cross-tolerance between [D-pen2, D-pen5] enkephalin and [D-ala2] deltorphin II in mice: evidence for delta receptor subtypes. *J Pharmacol Exp Ther* 258: 583-87, 1991.

38. **Musha T, Satoh E, Koyanagawa H, Kimura T, and Satoh S.** Effects of opioid agonists on sympathetic and parasympathetic transmission to the dog heart. *J Pharmacol Exp Ther* 250: 1087-1091, 1989.

39. **Noda M, Furutani Y, Takahashi H, Toyosato M, Hirose T, Inayama S, Nakanishi S, and Numa S.** Cloning and sequencing analysis of cDNA from bovine adrenal proenkephalin. *Nature*, 295:202-206, 1982.

40. **Opie LH.** Hypothesis: glycolytic rates control cell viability in ischemia. *J Appl Cardiol* 3: 407-414, 1988.

41. **Pokrovsky VM, and Oadchiy O.** Regulatory peptides as modulators of vagal influence on cardiac rhythm. *Can J Physiol Pharmacol* 73: 1235-1245, 1995.

42. **Portoghese PS, Sultana M, Nagase H, and Takemori AE.** A highly selective delta 1 receptor antagonist: 7 benzylidenenaltrexone. *Eur J Pharmacol* 218: 195-96, 1992.

43. **Schultz J el J, Hsu AK, Nagase H, and Gross GJ**. TAN-67 a delta 1 opioid receptor agonist, reduces infarct size via activation of Gi/o proteins and KATP channels. *Am J Physiol (Heart Circ Physiol)* 274: H909-H914, 1998.

44. **Schultz JE, and Gross GJ.** Opioids and cardioprotection. *Pharmacol Ther* 89 (2):123-37, 2001.

45. **Searles CD.** The nitric oxide pathway and oxidative stress in heart failure. Congest Heart Fail 8 (3): 142-7, 2002.

46. **Sears CE, Choate JK, and Paterson DJ.** NO-cGMP pathway accentuates the decrease in heart rate caused by cardiac vagal nerve stimulation. *J. Appl. Physiol.* 86: 510-516, 1999.

47. **Sofuoglu M, Portoghese PS, and Takemori AE**. Differential antagonism of delta opioid agonists by naltrindole and its benzofuran analog (NTB) in mice: evidence for delta opioid receptor subtypes. *J Pharmacol Exp Ther* 257: 676-80, 1991.

48. **Sofuoglu M, Portoghese PS, and Takemori AE**. Cross-tolerance studies in the spinal cord of beta FNA treated mice provides further evidence of delta opioid receptor subtypes. *Life Sci Pharmacol Lett* 49: PL153-56, 1991.

49. **Stefano GB, Salzet M, Hughes TK, and Bilfinger TV**. Delta II opioid receptor subtype on human vascular endothelium uncouples morphine stimulated nitric oxide release. *Int. J. Cardiol.* 30: 64 suppl 1: S43-51, 1998.

50. **Stewart PE, and Hammond DL**. Evidence for delta opioid receptors in rat spinal cord: studies with intrathecal naltriben, cyclic [D-pen2, D-pen5]-enkephalin and [D-ala2, Glu4] deltorphin. *J Pharmacol* 236: 89-96, 1993.

51. **Tanaka K, Okshima H, Hiroyasu E, and Tnemichi E**. Direct synaptic contacts of nitric oxide synthase-immunoreactive nerve terminals on the neurones of the intracardiac ganglia of the guinea pig. *Neurosci. Lett.* 158: 67-70, 1993.

52. **Tobin JR, Martin LD, Breslow MJ, and Traystman RJ**. Selective inhibition of brain nitric oxide synthase. *Anesthesiology* 81: 1264-9, 1994.

53. **Weitzell LR, Illes P, and Starke K.** Inhibition via opioid mu and delta receptors of vagal transmission in rabbit isolated heart. *Arch Pharmacol* 328:186 -190, 1984.

54. **Younes A, Pepe S, Barron BA, Spurgeon H, Lakatta, and Caffrey JL.** High rates of proenkephalin processing in the rat hart. *Am J Physiol* 279: H1989- H1998, 2000.

55. **Zaki PA, Bilsky EJ, Evans CJ, and Porreca F.** Opioid Receptor types and subtypes: the delta receptor as a model. *Annu Rev Pharmacol Toxicol* 36: 379-401, 1996.

56. **Zamotrinsky AV, Kondratiev B, and de Jong JW.** Vagal neurostimulation in patients with coronary artery disease. *Auton Neurosci* 88 (1-2): 109-16, 2001.

CHAPTER II

Cardiac Enkephalins Interrupt Vagal Bradycardia Via δ-2 Opioid Receptors in the Sinoatrial Node.

Martin Farias III, Keith E. Jackson, Darice Yoshishige, and James L. Caffrey.

University of North Texas Health Science Center

Department of Integrative Physiology

3500 Camp Bowie Boulevard

Fort Worth, Texas 76107

Running head: Vagolytic Opioid Receptors in the Sinoatrial Node

Corresponding author: James L. Caffrey

University of North Texas Health Science Center

Department of integrative Physiology

3500 Camp Bowie Boulevard

Fort Worth, TX 76107

817-735-2563

jcaffrey@hsc.unt.edu

Keywords: Vagal bradycardia, TAN-67, opioids and heart rate,

Abstract

Local cardiac opioids appear to be important in determining the quality of vagal control of heart rate. Introduction of the endogenous opioid, methionine enkephalin-arginine-phenylalanine (MEAP) into the interstitium of the canine sinoatrial node by microdialysis attenuates vagally mediated bradycardia through a δ-opioid receptor mechanism. The following studies were conducted to test the hypothesis that a δ-2 opiate receptor subtype mediates the interruption of vagal transmission. Twenty mongrel dogs were anesthetized and instrumented with microdialysis probes inserted into the sinoatrial node. Vagal frequency responses were performed at 1, 2 and 3 Hz during vehicle infusion and during treatment with the native agonist (MEAP) and assorted δ-1 opioid (TAN-67 and DPDPE) and δ-2 (deltorphin II) agonists. The vagolytic effects of intranodal MEAP and deltorphin were then challenged with the δ-1 and δ-2 opioid receptor antagonists BNTX and naltriben, respectively. Although the positive control, deltorphin II was clearly vagolytic in each experimental group, TAN-67 and DPDPE were vagolytically ineffective in the same animals. In contrast, TAN-67 improved vagal bradycardia by 30-35 percent. Naltriben completely reversed the vagolytic effects of MEAP and deltorphin. BNTX was ineffective in this regard but did reverse the vagal improvement observed with TAN-67. These data support the hypothesis that the vagolytic effect of the endogenous opioid MEAP was mediated by δ-2 opioid receptors located in the sinoatrial node. These data also support the existence of vagotonic δ-1 opioid receptors also in the sinoatrial node.

INTRODUCTION

The role of endogenous opioid peptides in the local control of heart rate is not yet well understood. When administered exogenously, these peptides are effective modulators of cardiac vagal function. Weitzell et al. (31) first reported that enkephalin inhibited vagal transmission in isolated rabbit hearts. The inhibition was reversed by the nonselective, opiate antagonist, naloxone. Other investigators have observed that enkephalins suppressed vagal bradycardia *in vivo* suggesting that enkephalins function as governors of vagal control (3,4,10,13,22,24).

Several enkephalin sequences are concentrated in the heart (32) including the heptapeptide, methionine-enkephalin-arginine-phenylalanine (MEAP). MEAP attenuated vagally mediated bradycardia by more than seventy percent when infused intra-arterially in anesthetized dogs and did not appear to involve a direct interaction with the pacemaker cells (3,4). The high affinity but nonselective opioid antagonist, diprenorphine completely reversed the effect of MEAP, restored vagal control of heart rate, and indicated that opiate receptors were involved (3,4).

Prejunctional vagal nerve terminals in the sinoatrial (SA) node and the nearby intracardiac parasympathetic ganglia were the most likely targets for MEAP. MEAP was delivered directly into the SA node by microdialysis to resolve these two potential targets. Intra-nodal MEAP attenuated vagally mediated bradycardia to the same extent as that observed during systemic infusion of the peptide. Both nodal and systemic effects were

reversed by the nodal delivery of diprenorphine (10). Collectively, these findings indicated that MEAP modulated vagal control of heart rate by acting on opioid receptors in the sinoatrial node which were most likely located prejunctionally on vagal nerve terminals.

In order to explore the physiology of opioids in the SA node, an extended series of dose response relationships with specific opioid agonists and antagonists were conducted to identify the responsible opioid receptor. Those studies have established a clear δ-receptor profile indicating that the vagolytic effect of MEAP was mediated by δ-opioid receptors (13). The nodal delivery of MEAP and the δ-2 agonist, deltorphin II produced equipotent vagolytic responses and both effects were reversed by the δ-antagonist, naltrindole. Mu and κ-agonists had no effect on vagally mediated bradycardia and μ- and κ-antagonists were ineffective versus MEAP (13). These data strongly indicated that δ-opioid receptors within the SA node were responsible for the vagolytic effect of MEAP.

Though distinct transcripts corresponding to δ-receptor subtypes have not been isolated (1,9,17), there is considerable functional and pharmacological evidence for the existence of distinct δ-1- and δ-2-receptor mediated responses (1,15,25,28,29,30,33). The nature of subtype specific actions on cardiac function is not well defined but Schultz et al (27) demonstrated that pretreatment with the selective δ-1-agonist, TAN-67 significantly reduced infarct size in the ischemic rat heart. The cardioprotection conferred by TAN-67

was subsequently reversed by the selective δ-1 antagonist, BNTX. Chien et al. (5) also reported that δ-1-agonists helped to preserve the viability of multi-organ preparations. Since the activation of cholinergic receptors has also been implicated in cardioprotection (34), a potential link between opioids and vagal function might be physiologically important. However, the vagolytic action of added MEAP cited above would be difficult to reconcile with reported cardioprotective effects of cholinergic stimulation.

The application of a preconditioning-like protocol to the SA node artery stimulated a reproducible increase in the endogenous MEAP recovered by dialysis from the nodal interstitium (14). In contrast to the vagolytic effect of exogenously administered MEAP, the rise in endogenous MEAP was accompanied by a consistent enhancement of vagally mediated bradycardia. The δ-antagonist, naltrindole, reversed the vagotonic effect and suggested participation by δ-opiate receptors (14). An opioid mediated increase in vagal function during arterial occlusion makes a role in cardioprotection mechanistically easier to explain. An increase in cholinergic stimulation during oxidative stress could reduce tissue loss by lowering metabolic demand locally.

These collected observations suggest the hypothesis that different subtypes of the δ-receptor (δ-1 and δ-2) may mediate respectively the opposing vagotonic and vagolytic effects of opioids. Consistent with the suggestion that the vagotonic effect is mediated by δ-1-receptors, Shultz et al (27) reported that TAN-67 reduced resting heart rate in the rat.

In contrast δ-activation by administered enkephalin in the dog produced a clear attenuation of vagal bradycardia. These opposing observations would be compatible if the vagolytic activity in the dog is mediated by δ-2 receptors. The two subtypes of δ-receptors may serve distinctly different roles in the regulation of heart rate.

The purpose of these studies was to test the hypothesis that δ-2 opioid receptors in the sinoatrial node were responsible for the vagolytic effect of the cardiac opioid MEAP and to rule out the participation of δ-1 opioid receptors. This was accomplished with two strategies. In one, the vagolytic effects of MEAP and the δ-2 agonist, deltorphin II were first demonstrated and then the endogenous opioid, MEAP was challenged with δ-1 and δ-2 selective antagonists. In the second, the vagolytic effects of MEAP and deltorphin II were compared with those of the selective δ-1 agonists, DPDPE and TAN-67. This endeavor arose as a result of previous studies, which established a role for δ-receptors in the vagolytic actions of MEAP. The efficacy of deltorphin II in those studies suggested the vagolytic effect might involve a δ-2 response, but the definitive comparisons were not available.

METHODS

Experiments conformed to the *Guide for the Care and the Use of Laboratory Animals* published by the National Institutes of Health.

Surgical preparation. Twenty Mongrel dogs were anesthetized with sodium pentobarbital, intubated and mechanically ventilated with room air. Fluid filled catheters were inserted into the femoral artery and vein then advanced into the descending aorta and inferior vena cava, respectively. The arterial line was attached to a Statham PD23XL pressure transducer to monitor heart rate and blood pressure continuously online (Powerlab). The venous line was used to administer additional anesthetic as needed. Arterial blood gases were monitored (Instrumentation Laboratories blood gas analyzer) and the pO_2 (90-120 mmHg), pH (7.35 -7.45), and pCO_2 (35-45 mmHg) were adjusted to normal with supplemental oxygen, bicarbonate, or by altering the minute volume.

The right and left vagus nerves were isolated in the cervical region through a midline surgical incision, tied off tightly with umbilical tape and returned to their position in the neck for later retrieval. A single dose of succinylcholine (1 mg/kg) was administered intravenously to temporarily reduce involuntary muscle movements during the 10-15 minutes required for the electrosurgical incision of the right thorax and removal of right ribs 2-5. The pericardium was opened and the upper margins were sutured to the body wall to provide a pericardial cradle.

A 27-gauge stainless steel cannula was used to introduce the microdialysis probe into the sinoatrial node. To confirm the probe placement in the SA node, norepinephrine (1 x 10^{-9} moles/μl) was introduced into the microdialysis probe. The observation of a brisk 30-40 beat increase in heart rate provided a functional confirmation of the probe location within the SA node. Prior studies have determined that deliberate repositioning of the probe as little as 2 mm lateral to the node eliminates the norepinephrine mediated tachycardia (14). The microdialysis probe was constructed from a single one centimeter length of dialysis fiber (220 μm OD, 200 μm ID) and hollow silica inflow and outflow tubes (120 μm ID, 170 μm OD). The dialysis tubing permits molecules with a molecular weight of 36,000 or less to freely cross from the lumen into the nodal interstitium. This technique enables sampling and manipulation of the local nodal interstitial environment while minimizing alterations in systemic hemodynamics and reflex compensations.

Protocols. These experiments were conducted to demonstrate that the δ-2 opioid receptor subtype was responsible for the vagolytic effect of nodal enkephalins. Two strategies were employed. In the first the influence of δ-subtype specific agonists [D-pen^{2,5}] enkephalin (DPDPE), TAN-67 and deltorphin II were compared for their vagolytic action. In the second strategy, a vagolytic effect of the endogenous agonist MEAP was established and then the ability of subtype selective antagonists (7-benzyldenenaltrexone [BNTX] and naltriben) to reverse this effect were evaluated. All treatments were

introduced locally into the interstitium of the SA node by microdialysis at a flow rate of 5 μl/min.

Previous studies revealed that the δ-2 selective agonist, deltorphin II (1.5×10^{-9} moles/min) blocked vagally mediated bradycardia (13). The vagolytic effect of deltorphin II was successfully reversed by the δ-selective antagonist, naltrindole. These findings suggested participation of a δ-2 opioid receptor in this effect. This study determined the subtype of δ-opioid receptor responsible for the inhibition of vagally mediated bradycardia by MEAP.

Protocol 1. This protocol tested whether the intra-nodal administration of δ-1-selective agonists is capable of interrupting vagal bradycardia. After microdialysis probe insertion, the SA node was perfused (5μl/min) with saline for 60 minutes. After this period of equilibration, control vagal responses were obtained by stimulating the right vagus nerve at 1,2, and 3 hertz. The nerve was stimulated at a supramaximal voltage for 15 seconds followed by one min 45 sec for recovery. Deltorphin II was then infused (5μl/min) into the sinoatrial node for five minutes to establish a functional vagolytic effect. The effective dose used for deltorphin II (1.5×10^{-9} moles/min) was determined previously (13). Once established, the vagolytic effect of deltorphin II served as a positive control in cases where the subsequent agonists under evaluation were without effect. Following this procedure, dose responses were constructed for the selective δ-1 agonist DPDPE or TAN-

67. Doses were selected to provide molar equivalent ranges (0.05 – 5 x 10^{-9} moles/min) to those previously determined to be vagolytic for MEAP and deltorphin II (13). Each dose of each agent was infused for five minutes before evaluating the vagus nerve. After each dose evaluation, the agent was washed out for 15 minutes and vagal function was retested to ensure that it had returned to normal. The length of washout was based on previous experiments (13). At the end of the TAN-67 protocol, this agent was combined with the δ-1 antagonist, BNTX, to determine if the unexpected improvement in vagal function was mediated by a δ-1 opioid receptor.

Protocol 2. This protocol was designed to test whether vagolytic effects of MEAP and deltorphin II are blocked by a selective δ-2 opioid receptor antagonist and not by a selective δ-1 opioid receptor antagonist. MEAP and deltorphin II (1.5 x 10^{-9} moles/min) were introduced into the interstitium of the sinoatrial node and vagal stimulations were performed as previously described in order to establish the vagolytic effect of each. After washout of these initial tests, MEAP was combined with increasing doses of the selective δ-1 antagonist, BNTX, or the selective δ-2 antagonist, naltriben. At the end of the protocol, the specific subtype was further confirmed by combining deltorphin II with the maximum effective dose of one or the other antagonist. The hypothesis predicts that the δ-2 antagonist, naltriben should overcome the vagolytic effect of MEAP and deltorphin and verify participation of the δ-2 opioid receptor. BNTX should not reverse the

vagolytic effect of MEAP or deltorphin indicating the absence of participation by δ-1 opioid receptors.

Materials. Methionine enkephalin-arginine-phenylalanine and deltorphin II were synthesized by American Peptide Co. TAN-67, DPDPE and BNTX were obtained from Tocris. Naltriben was obtained from Sigma Chemical Co.

Statistical methods. All data were expressed as means and standard errors. Differences were evaluated with ANOVA for repeated measures. Individual treatment differences were determined by post hoc analysis with Tukey's test for multiple comparisons. Differences determined to occur by chance with a probability of $p < 0.05$ were accepted as statistically significant.

RESULTS

Twenty dogs were randomly assigned to various protocols employing δ-1- and δ-2-agonists and antagonists. Table 1 represents the resting cardiovascular parameters for all animals across all treatments. There were no significant differences in heart rate or blood pressure among groups prior to treatment. Resting heart rate and blood pressure were also unaltered by any of the opioid agonists and antagonists, regardless of dose.

Deltorphin vagolysis. Deltorphin II was used as a positive control to demonstrate the functional integrity of the system in each animal prior to testing other agents. This pretest also served to verify the appropriate placement of the dialysis probe in the proximity of

the nodal opiate receptors responsible for the interruption of vagal bradycardia. The nodal administration of deltorphin II (1.5×10^{-9} moles/min) reduced vagally mediated bradycardia by 75-85 percent at all vagal frequencies employed and was significantly different from control.

DPDPE dose responses: In this protocol DPDPE was introduced directly into the sinoatrial node to rule out the participation of δ-1 opioid receptors in the opioid mediated interruption of vagal bradycardia. Control vagal stimulations during vehicle infusion produced a normal graded decline in heart rate at all vagal frequencies used (Figure 1). The nodal delivery of DPDPE had no effect on heart rate during the vagal frequency response as indicated by the superimposition of the DPDPE and vehicle responses (lower two curves). The vagolytic effect of deltorphin II is illustrated in the upper curve. The complete dose responses for all three frequencies are illustrated in Figure 2.

TAN-67 dose responses: In the absence of an effect as observed with DPDPE, it is difficult to say with confidence that the agent successfully crossed the dialysis membrane into the interstitium. In this regard a second selective δ-1 opioid receptor agonist, TAN-67 was used in a second group of animals to provide further evidence that δ-1 opioid receptors were not vagolytic. During vehicle infusions, control vagal stimulations produced a normal graded decline in heart rate as the frequency of stimulation was increased (Figure 3, middle curve). Deltorphin II produced a vagolytic response similar

to that observed (80% inhibition) in the prior group (Figure 3, upper curve). The administration of TAN-67 into the SA node had no vagolytic effect during the vagal frequency response at any dose employed. Rather, TAN-67 produced a greater vagal bradycardia as the dose was increased (Figure 3, lower curve). The maximum effect was observed at the 1.5×10^{-9} moles/min (Figure 4) with an apparent ED50 of 0.1×10^{-9} moles/min. The maximal improvement at 1.5×10^{-9} moles/min was 28-37 percent and was significantly different from control at all vagal frequencies.

Acting on the presumption that the vagotonic effect of TAN-67 was perhaps mediated by a δ-1 receptor, TAN-67 (1.5×10^{-9} moles/min) was then combined with the δ-1 antagonist BNTX (1.5×10^{-9} moles/min) and infused directly into the sinoatrial node via microdialysis. BNTX effectively prevented the vagotonic effect of TAN-67 since the vagally mediated bradycardia during the combined infusion was similar to control values (Fig 3, middle curve). The administration of BNTX alone had no effect on vagal bradycardia and once again produced values that were similar to control. Vagal stimulations were performed after washout of each treatment and were again similar to control values.

MEAP vs Naltriben dose responses: In the second strategy, deltorphin II and the endogenous cardiac opioid, MEAP were introduced into the SA node at vagolytically effective doses. Each agonist was subsequently combined with selective δ-1 and δ-2 antagonists to verify which δ-receptor subtype was responsible for the interruption of

vagal bradycardia. The control frequency response is illustrated among the lower curves in Figure 5. The vagolytic effect of deltorphin II and MEAP are illustrated in the two upper curves. Increasing doses of the selective δ-2, opioid receptor antagonist, naltriben were combined with MEAP in the dialysis perfusate. Naltriben progressively reversed the effect of MEAP and restored vagal regulation of heart rate to control (Figure 6). The reversal was obtained with an ID50 approximating 1.5×10^{-10} moles/min and a maximal effect near molar parity with the agonist (1.5×10^{-9} moles/min). The similar blockade of the deltorphin and MEAP effects are illustrated among the lower curves in Figure 5 for the last dose in the naltriben dose response curve. Perfusion with the highest dose of naltriben alone was similar to control indicating that naltriben had no effect on vagal function independent of its ability to obstruct the access of MEAP and deltorphin II to nodal δ-2 receptors.

MEAP vs BNTX dose responses: The selective δ-1-opioid receptor antagonist BNTX was used to confirm that the vagolytic effect of MEAP was mediated by δ-2- and not by δ-1-opioid receptors. This was achieved by combining increasing doses of BNTX with an effective vagolytic dose of MEAP (1.5×10^{-9} moles/min). The rationale presumed that if naltriben identified a functional δ-2-response, then combining MEAP with increasing doses of BNTX would find BNTX ineffective or much less effective than naltriben. The lower two curves in Figure 7 illustrate the control bradycardia response in this group and the absence of an effect of BNTX alone. The 50-70 percent inhibition by both MEAP and deltorphin II are indicated among the upper curves in Figure 7. When BNTX was

combined with MEAP or deltorphin II, the resulting curves were very similar to those for MEAP and deltorphin alone (Figure 7, upper curves). BNTX had no effect on the vagolytic properties of either MEAP or deltorphin. The complete dose response curves for BNTX versus MEAP are described in Figure 8. Though a subtle reversal of the effect of MEAP might be suggested from these data, the observed bradycardia was never different from MEAP alone. The absence of an effect of BNTX versus both MEAP and the δ-2-agonist, deltorphin II further supports the exclusive δ-2-character of the vagolytic effect.

DISCUSSION

The data reported above support the primary hypothesis that the vagolytic effect of the endogenous opioid, MEAP on heart rate is mediated by δ-2-opioid receptors in the sinoatrial node. This conclusion is based on the observation that the vagolytic effect of MEAP was duplicated by the δ-2 agonist deltorphin II when the δ-1-agonists, DPDPE and Tan-67 were both vagolytically ineffective in the same animals. Participation by δ-2-receptors was verified further by demonstrating the vagolytic effect of MEAP was reversed by the δ-2-antagonist, naltriben and unaltered by equimolar doses of the δ-1-antagonist, BNTX. The δ-character of the vagolytic effect of MEAP was rigorously determined earlier (13) and the current findings suggest that the vagolytic effect was mediated by δ-2-receptors without a measurable δ-1-receptor contribution.

Deltorphin II served as positive control in these experiments to confirm the location of the dialysis probe within functional reach of the nodal opioid receptors responsible for the vagolytic response. The absence of a response when introducing agents by microdialysis can be ambiguous because it is often difficult to verify that every agent has successfully crossed the dialysis membrane into the interstitium in biologically effective concentrations. In this instance, functionally similar but molecularly distinct δ-1-agonists were used to reduce the probability of interference with diffusion due to molecular charge, adsorption, or solubility. In this case, both DPDPE and TAN-67 are δ-1-agonists but DPDPE is a modified peptide and TAN-67 is a heterocyclic isoquinoline. The use of both agents dramatically reduces the probability that the absence of a δ-1-effect resulted from failure of agents to reach the target due to adsorption or failure to diffuse freely.

Although TAN-67 had no vagolytic effect, it produced a consistent improvement in vagal bradycardia and thus provided additional direct evidence that TAN-67 had reached the nodal interstitium. The δ-1-opioid receptor antagonist, BNTX subsequently reversed the TAN-67 mediated vagal improvement. Thus δ-1- receptors were present in the SA node and were vagotonic rather than vagolytic. These observations suggested that the opioid modulation of vagal function is bimodal with opposite poles of the response mediated by different subtypes of the δ-receptor.

Selectivity issues: TAN-67 and DPDPE. The existence of δ receptor subtypes has been based entirely on biological responses which can be distinguished by agonists and antagonists reported as selective for the respective subtypes (1,13,25,28,29,30,33). Each receptor subtype stimulated responses that were reversed by agonists preferential to that subtype. Mixed results were obtained when cross-tolerance or cross-desensitization experiments were conducted (1,21,29). A single receptor transcript has been isolated and attempts to identify distinct receptor proteins associated with δ-1- and δ-2-mediated responses have been as yet unsuccessful (1,9,17). Contradictory findings in some isolated systems *in vitro* support the suggestion that differences in coupling, agonist concentration or local membrane conditions may determine whether δ-1-, δ-2-, or mixed responses are evident (7).

Subtype specific responses have been used to quantify the relative δ-selectivity of various agents. DPDPE and deltorphin II have been widely employed respectively as preferential δ-1- and δ-2-agonists. Each has approximately 80 to 100-fold selectivity for its respective receptor subtype in antinociceptive and binding studies (6,8,30). Antagonists for each receptor subtype have been characterized as well. BNTX and naltriben currently serve respectively as prototypical δ-1- and δ-2-antagonists (15,25).

DPDPE reportedly has some mixed δ-2-agonist activity in some biological systems (33). This aspect might complicate the interpretation of the absent response with DPDPE during vagal stimulations and may help to explain the difference observed between

DPDPE and TAN-67. Since δ-2-opioid receptors were clearly vagolytic, the absence of a response to DPDPE would suggest either the absence of δ-1-receptors or the absence of a δ-1-effect on vagal function. If DPDPE has measurable δ-2-activity, one might expect to see a vagolytic response at the high end of the dose response curve. TAN-67 which is significantly more selective for δ-1-opioid systems (6,16) augmented vagal bradycardia by 35 percent and was reversed by BNTX. This suggests that δ-1-receptors were present and they did alter vagal function through an apparent δ-1-mechanism. If DPDPE acted on both δ-1- and δ-2-receptors simultaneously, opposing vagotonic and vagolytic actions may have cancelled out one another. In summary, selective activation of δ-1-receptors had no demonstrable vagolytic effect. In contrast, δ-1-receptors appear to facilitate vagal function.

The normal role of cardiac opioids in the autonomic control of the heart remains unclear but some of the details have begun to resolve. The presence of significant mRNA for proenkephalin in heart and the heart's prodigious capability to degrade enkephalin suggest the cardiac enkephalins function primarily as local paracrine hormones. The studies reported here have concentrated on interactions with vagal control of heart rate. Earlier studies both *in vivo* and in isolated heart models demonstrated that opioids attenuated a variety of cardiac parasympathetic responses during vagal nerve stimulation (3, 4, 10, 13, 22, 24, 31). The δ-2-mediated interruption of vagal bradycardia is consistent with the traditional view of opioids as inhibitory neuromodulators. The apparent bimodal character of δ-receptor activation though not often acknowledged is

also not that unusual (7,26). Since distinct δ-1- and δ-2-receptor proteins have not been isolated, opposing responses in the same tissue presents some interesting mechanistic questions. One proposal suggested that the local membrane environment determined the functional expression of opposing opioid receptor responses by regulating how the receptors were coupled to their respective second messenger systems (7). How this local environment and the balance of these responses participate in normal heart rate control remains to be determined.

What purpose do these δ-subtypes serve in modulating heart rate during normal homeostasis? When endogenous nodal MEAP was elevated during occlusion of the nodal artery, vagal bradycardia was improved (14). The vagotonic effect was blocked by the general δ-antagonist, naltrindole and the vagal improvement was quantitatively very similar to that observed during administration of TAN-67 in this current report. Since the latter was blocked by BNTX, both responses may have been mediated by δ-1-receptors. The coupling hypothesis cited above (7) also suggested that one side of the bimodal response was far more sensitive to agonist. The hypothesis argued that the positive coupling to adenylate cyclase through the G-protein, Gsα predominated at physiologically very low opioid concentrations. Thus the vagotonic effect associated with nodal artery occlusion would be consistent with the bimodal hypothesis if the modest increases in nodal MEAP also observed during occlusion (14) improved the efficiency of vagal transmission through δ-1-receptors much like TAN-67. The activation of δ-1-receptors

during arterial insufficiency might serve to stabilize the heart by improving local vagal function and thereby reducing local oxygen demand and consequent irritability.

At the other end of the spectrum, vasovagal syncope poses a different threat to the organism during stressful circumstances. In this regard, higher rates of opioid release combined with the activation of δ-2-opioid receptors may suppress vagal function when that activity is inappropriately intense. Thus at higher concentrations the more widely recognized neuroinhibitory coupling to adenylate cyclase through the inhibitory G-protein Glα might predominate with the opioids now serving as inhibitory governors of vagal activity. In accord with this proposed hypothesis, one might argue that the δ-1-activity provides a background environment of neurofacilitatory activity while the δ-2-receptors provide a more episodic "governor-like" function.

The opioid receptor systems may also be of significance during cardiovascular pathologies such as myocardial infarction and congestive heart failure. Evidence that δ-1-receptors mediate preconditioning suggested that these receptors might be therapeutically valuable during myocardial infarction (27). Conducting preconditioning-like protocols with the sinoatrial node artery demonstrated that nodal MEAP was elevated during repeated arterial occlusion. As indicated above, this increase in nodal MEAP was accompanied by an improved vagal function (14) that in retrospect may have been mediated by δ-1-receptors. Healthy vagal influences have been associated with better

survival statistics after myocardial infarction (2, 18). The activation of δ-1-receptors could enhance vagal function during myocardial infarction and by slowing the heart, decrease work output and energy demand (23, 34). This would then reduce the damage caused by free radicals and help to maintain cellular integrity (23).

The observation that δ-2-opioid receptors are vagolytic suggests that their actions may be pathologic during sustained excess. Circulating endogenous opioids rise significantly during congestive heart failure (11). The vagolytic action of these peptides may contribute to cardiac dysfunction and the rise in sympathetic activity. In support of this hypothesis, δ-opioid antagonists restored vagal function in atrial preparations from failing human hearts (19). However, the characterization of δ-1- and δ-2-receptor effects on heart rate during cardiovascular disease remains to be elucidated and may hold significant clinical potential.

Conclusions. In conclusion, the current results suggested that the endogenous cardiac enkephalin, MEAP, attenuated vagal bradycardia via δ-2-opioid receptors concentrated within the canine sinoatrial node. The data above also support the presence of δ-1-opioid receptors in the SA node that appear to facilitate vagal transmission. Whether δ-1- and δ-2-opioid receptors in the SA node are located prejunctionally on vagal nerve terminals and whether these receptors modify the release of acetylcholine both remain to be verified directly and as such constitute important future directions.

REFERENCES

1. **Allouche S, Hasbi A, Ferey V Sola B, Jauzak P, and Polastron J.** Pharmacological δ-1- and δ-2-opioid receptor subtypes in the human neuroblastoma cell line SK-N-BE: No evidence for distinct molecular entities. *Biochem Pharmacol* 59: 915-925, 2000.

2. **Billman GE, Schwartz PJ, and Stone HL.** The effects of daily exercise on the susceptibility to sudden cardiac death. *Circulation* 69: 1182-1189, 1984.

3. **Caffrey JL, Mateo Z, Napier LD, Gaugl JF, and Barron BA.** Intrinsic cardiac enkephalins inhibit vagal bradycardia in the dog. *Am J Physiol (Heart Circ Physiol* 37) 268: H848-H855, 1995.

4. **Caffrey JL.** Enkephalin inhibits vagal control of heart, contractile force, and coronary blood flow in the dog in vivo. *J Auto Nerv Syst* 2318: 1-8, 1999.

5. **Chien S, Oeltgen PR, Diana JN, and Salley RK.** Extension of survival time in multi organ block preparation with a δ-opioid DADLE ([D-Ala2, D-Leu5]-enkephalin). *J Thorac Cardiovascular Surg* 107: 964-967, 1994.

6. **Clarke GD, and Dondio GM.** Recent advances in the development of a non-peptide δ-opioid analgesic. *Pharmacol Rev Commun* 9: 49-60, 1997.

7. **Crain SM, and Shen KF.** Modulation of opioid analgesia, tolerance and dependence by Gs-coupled, GM-1 ganglioside-regulated opioid receptor functions. (Review). *TIPS* 19: 358-365, 1998.

8. **Erspamer V, Melchiorri P, Flaconieri-Erspamer G, Negri L, and Corsi R.** Deltorphins: a family of naturally occurring peptides with a high affinity and selectivity for δ-opioid binding sites. *Proc Natl Acad Sci USA* 86: 5188-92, 1989.

9. **Evans CJ, Keith DE, Morrison H, Magendzo K, and Edwards RH.** Cloning of a δ-opioid receptor by functional expression. *Science* 258: 1952-1955, 1992.

10. **Farias M, Jackson K, Stanfill A, and Caffrey JL.** Local opiate receptors in the sinoatrial node moderate vagal bradycardia. *Auto Neurosci (Basic and Clin)* 87: 9-15, 2001.

11. **Fontana F, Bernardi P, Pich EM, Capelli M, Bortoluzzi L, Spampinato S, and Canossa M.** Relationship between plasma atrial natriuretic factor and opioid levels in

healthy subjects and in patients with acute congestive heart failure. *Eur Heart J* 14: 219-225, 1993

12. **Goldstein A, and Naidu A.** Multiple opioid receptors: ligand selectivity profiles and biding signatures. *Mol Pharmacol* 36: 265-72, 1989.

13. **Jackson KE, Farias M, Stanfill A, and Caffrey JL.** Delta opioid receptors inhibit vagal bradycardia in the sinoatrial node. *J Cardiovasc Pharmacol Therapeut* 6(4): 385-393, 2001.

14. **Jackson KE, Farias M, Stanfill, and Caffrey JL.** Transient arterial occlusion raises enkephalin in the sinoatrial node and improves vagal bradycardia. *Auto Neurosci* (Basic and Clin) 94: 84-92, 2001.

15. **Jiang Q, Takemori AE, Sultana M, Portoghese PS, and Bowen WD.** Differential antagonism of δ opioid antinociception by [D-ala2, Leu5, Cys6]-enkephalin and 5'-naltrindole isothiocyanate (5'-NTII): evidence for δ-receptor subtypes. *J Pharmacol Exp Ther* 257: 1069-75, 1991.

16. **Kamei J, Saitoh A, Ohsawa M, Suzuki T, Misawa M, Nagase H, and Kasuya Y.** Antinociceptive effects of the selective non-peptidic δ-opioid receptor agonist TAN-67 in diabetic mice. *Eur J Pharmacol* 276: 131-135, 1995.

17. **Kieffer BL, Befort K, Gaveriaux-Ruff c , a nd H irth C G.** the δ-opioid receptor: Isolation of a cDNA by expression cloning and pharmacological characterization. *Proc Natl Acad Sci* 89: 12048-12052, 1992.

18. **Kleiger RE, Miller LP, and Bigger JT**. Decreased heart rate variability and its association with increased mortality after acute myocardial infarction. *Am J Cardiol* 59: 256-262, 1987.

19. **Llobel F , a nd L aorden M L**. Effects of μ-, δ- and κ- opioid antagonists in atrial preparations from nonfailing and failing human hearts. *Gen Pharmacol* 28: 371-374, 1997.

20. **Martin WR.** Pharmacology of opioids. *Pharmacol Rev* 35: 285-323, 1984.

21. **Mattia A, Vanderah T, Mosberg HI, and Porreca F**. Lack of antinociceptive cross-tolerance between [D-pen2, D-pen5] enkephalin and [D-ala2] deltorphin II in mice: evidence for δ-receptor subtypes. *J Pharmacol Exp Ther* 258: 583-87, 1991.

22. **Musha T, Satoh E, Koyanagawa H, Kimura T, and Satoh S.** Effects of opioid agonists on sympathetic and parasympathetic transmission to the dog heart. *J Pharmacol Exp Ther* 250: 1087-1091, 1989.

23. **Opie LH.** Hypothesis: glycolytic rates control cell viability in ischemia. *J Appl Cardiol* 3: 407-414, 1988.

24. **Pokrovsky VM, and Oadchiy O.** Regulatory peptides as modulators of vagal influence on cardiac rhythm. *Can J Physiol Pharmacol* 73: 1235-1245, 1995.

25. **Portoghese PS, Sultana M, Nagase H, and Takemori AE.** A highly selective δ-1-receptor antagonist: 7-benzylidenenaltrexone. *Eur J Pharmacol* 218: 195-96, 1992.

26. **Sarne Y, Fields A, Keren O, and Gafni M.** Stimulatory effects of opioids on transmitter release and possible cellular mechanisms: overview and original results. (Review) *Neurochemical Research.* 21:1353-61, 1996.

27. **Schultz J el J, Hsu AK, Nagase H, and Gross GJ.** TAN-67 a δ-1-opioid receptor agonist, reduces infarct size via activation of Gi/o proteins and KATP channels. *Am J Physiol (Heart and Circ Physiol)* 274: H909-H914, 1998.

28. **Sofuoglu M, Portoghese PS, and Takemori AE.** Differential antagonism of δ-opioid agonists by naltrindole and its benzofuran analog (NTB) in mice: evidence for δ-opioid receptor subtypes. *J Pharmacol Exp Ther* 257: 676-80, 1991.

29. **Sofuoglu M, Portoghese PS, and Takemori AE.** Cross-tolerance studies in the spinal cord of beta FNA treated mice provides further evidence of δ-opioid receptor subtypes. *Life Sci Pharmacol Lett* 49: PL153-56, 1991.

30. **Stewart PE, and Hammond DL.** Evidence for δ-opioid receptors in rat spinal cord: studies with intrathecal naltriben, cyclic [D-pen2, D-pen5]-enkephalin and [D-ala2, Glu4] deltorphin. *J Pharmacol* 236: 89-96, 1993.

31. **Weitzell LR, Illes P, and Starke K.** Inhibition via opioid μ- and δ-receptors of vagal transmission in rabbit isolated heart. *Arch Pharmacol* 328:186 -190, 1984.

32. Younes A, Pepe S, Barron BA, Spurgeon H, Lakatta, Caffrey JL. High rates of proenkephalin processing in the rat hart. *Am J Physiol* 279: H1989- H1998, 2000.

33. **Zaki PA, Bilsky EJ, Evans CJ, and Porreca F.** Opioid Receptor types and subtypes: the δ-receptor as a model. *Annu Rev Pharmacol Toxicol* 36: 379-401, 1996.

34. **Zamotrinsky AV, Kondratiev B, and de Jong JW.** Vagal neurostimulation in patients with coronary artery disease. *Auton Neurosci* 88 (1-2): 109-16, 2001.

LEGEND

FIG.1 This graph represents the heart rate/ frequency response mediated by right vagal nerve stimulation during the nodal delivery of Deltorphin II (1.5×10^{-9} moles/min) and DPDPE (5×10^{-9} moles/min) by microdialysis. The data illustrated are for the maximal dose of DPDPE employed in its dose response curve. * = Significantly different from control, $P < .05$.

Fig 2 (a,b,c). These graphs illustrate the change in heart rate produced during right vagal stimulation during exposure (5 min) to increasing doses of the δ-1-selective opioid agonist, DPDPE. The units for the doses listed within the bars are x 10^{-9} moles/min. Deltorphin (1.5×10^{-9} moles/min) was included as a positive control. All treatments were infused into the sinoatrial node of the dog via microdialysis. * = Significantly different from control, $P < .05$.

Fig 3. This graph represents the heart rate/ frequency response mediated by right vagal nerve stimulation during the nodal delivery of vehicle, deltorphin II (1.5×10^{-9} moles/min), TAN-67 (5×10^{-9} moles/min) or BNTX alone (5×10^{-9} moles/min), and TAN-67 (1.5×10^{-9} moles/min) combined with an equimolar dose of BNTX and BNTX alone. * = Significantly different from control , $P < .05$.

Fig 4 (a,b,c). These graphs illustrate the change in heart rate produced during right vagal stimulation during exposure (5 min) to increasing doses of the δ-1-selective opioid

agonist, TAN-67. The units for the doses listed within the bars are x 10^{-9} moles/min. Deltorphin (1.5 x 10^{-9} moles/min) was included as a positive control. All treatments were infused into the sinoatrial node of the dog via microdialysis. * = Significantly different from control, P< .05.

Fig 5. This graph represents the heart rate/ frequency response mediated by right vagal nerve stimulation during the nodal delivery of vehicle, deltorphin II (1.5 x 10^{-9} moles/min), MEAP (1.5 x 10^{-9} moles/min), MEAP or deltorphin II (1.5 x 10^{-9} moles/min) combined with an equimolar dose of Naltriben, and Naltriben (5 x 10^{-9} moles/min) alone. * = Significantly different from control, P< .05.

Fig 6 (a,b,c). These graphs illustrate the change in heart rate produced during right vagal stimulation during exposure (5 min) to increasing doses of the δ 2 antagonist, naltriben combined with MEAP (1.5 x 10^{-9} moles/min). The units for the doses listed in the bars are x 10^{-9} moles/min. Deltorphin (1.5 x 10^{-9} moles/min) was included as confirmation of the δ-2-character of the naltriben blockade. All treatments were infused into the sinoatrial node of the dog via microdialysis. * = Significantly different from control, P< .05.

Fig 7. This graph represents the heart rate/ frequency response mediated by right vagal nerve stimulation during the nodal delivery of vehicle, deltorphin II (1.5 x 10^{-9} moles/min), MEAP (1.5 x 10^{-9} moles/min), MEAP or deltorphin II (1.5 x 10^{-9} moles/min)

combined with an equimolar dose of BNTX, and BNTX (5 x 10^{-9} moles/min) alone. * = Significantly different from control, P< .05.

Fig 8 (a,b,c). These graphs illustrate the change in heart rate produced during right vagal stimulation during exposure (5 min) to increasing doses of the δ-1-antagonist, BNTX combined with a fixed dose of MEAP (1.5 x 10^{-9} moles/min). The units for the doses listed in the bars are x 10^{-9} moles/min. Deltorphin (1.5 x 10^{-9} moles/min) was included as added confirmation of the absent δ-1 receptor participation in the response. All treatments were infused into the sinoatrial node of the dog via microdialysis. * = Significantly different from control, P< .05.

Table 1. Cardiovascular Indices

Groups (n)	Control		Treatment		Washout	
	HR	MAP	HR	MAP	HR	MAP
MEAP (15)	128±5	114±7	132±7	113±7	125±5	114±8
Delt (13)	127±4	118±4	128±6	114±6	126±4	112±9
DPDPE (5)	129±5	112±7	127±5	109±7	128±4	112±6
TAN-67 (5)	122±6	117±7	110±4	119±9	117±3	114±8
BNTX (5)	125±4	117±5	111±2	108±9	121±4	109±7
Naltriben (5)	136±7	112±7	123±6	118±5	120±2	114±4

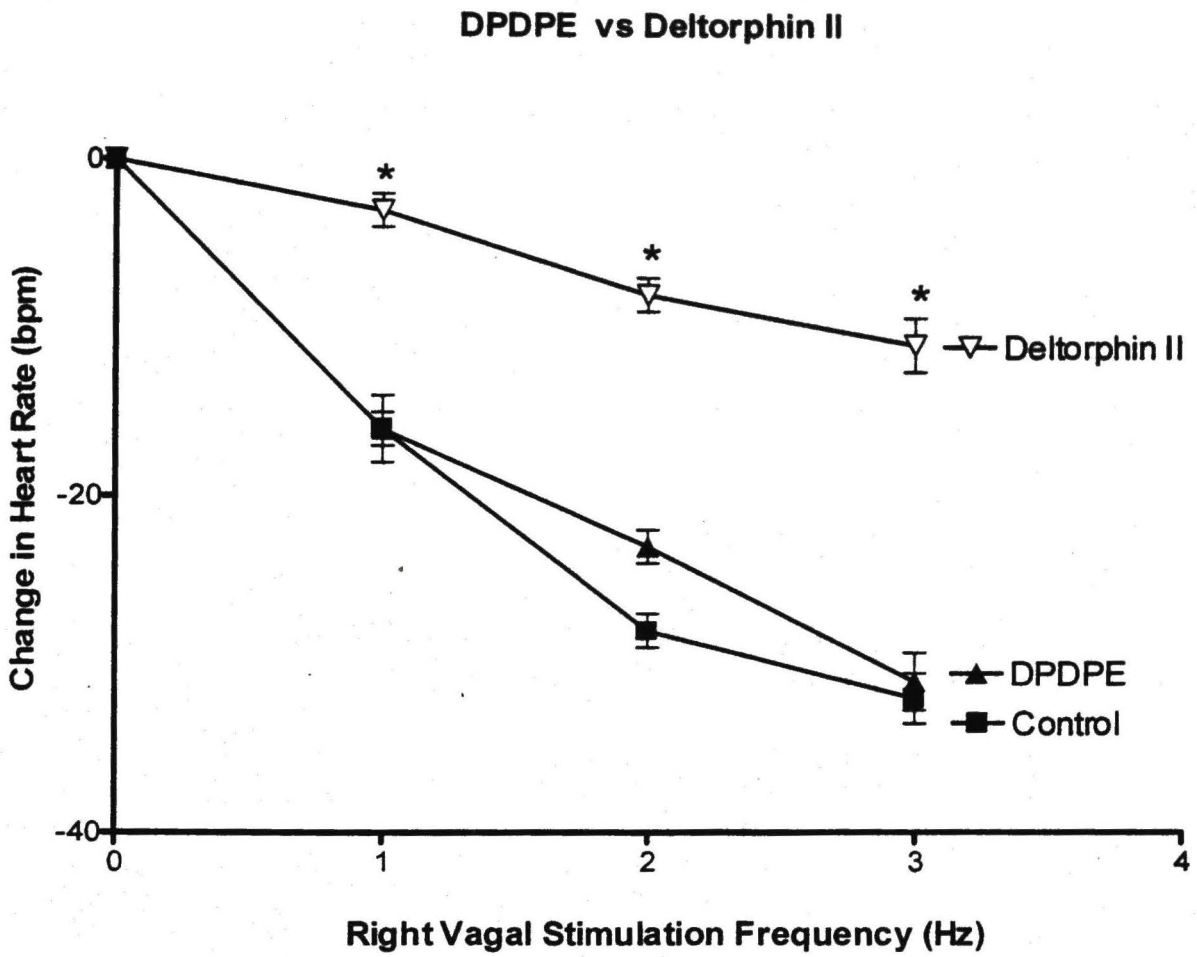
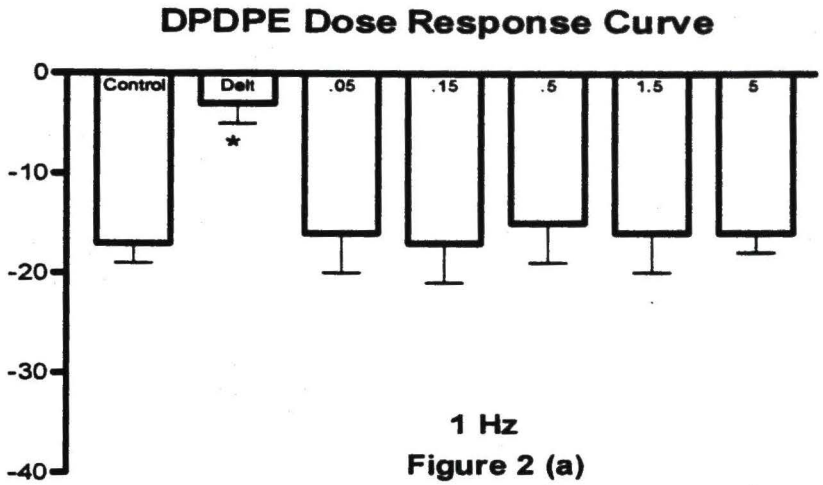
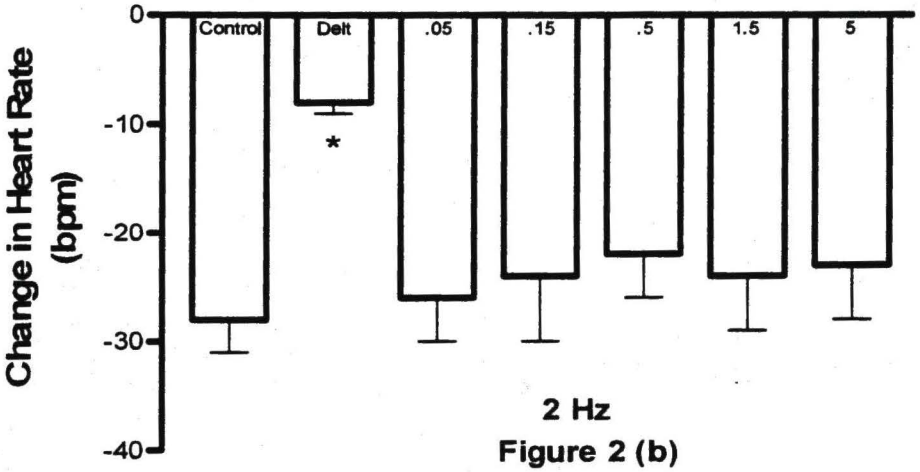

DPDPE vs Deltorphin II
0
-20
-40
Change in Heart Rate (bpm)
*
*
*
Deltorphin II
DPDPE
Control
0
1
2
3
4
Right Vagal Stimulation Frequency (Hz)

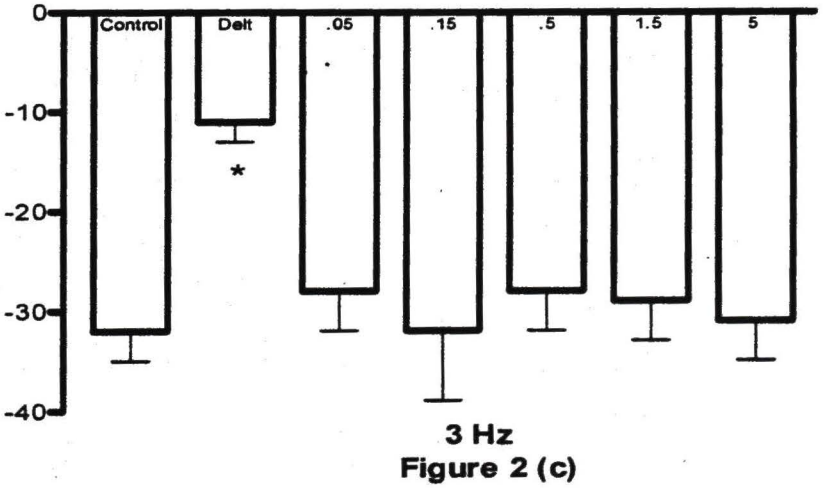
Figure 1



1 Hz
Figure 2 (a)



2 Hz
Figure 2 (b)



3 Hz
Figure 2 (c)

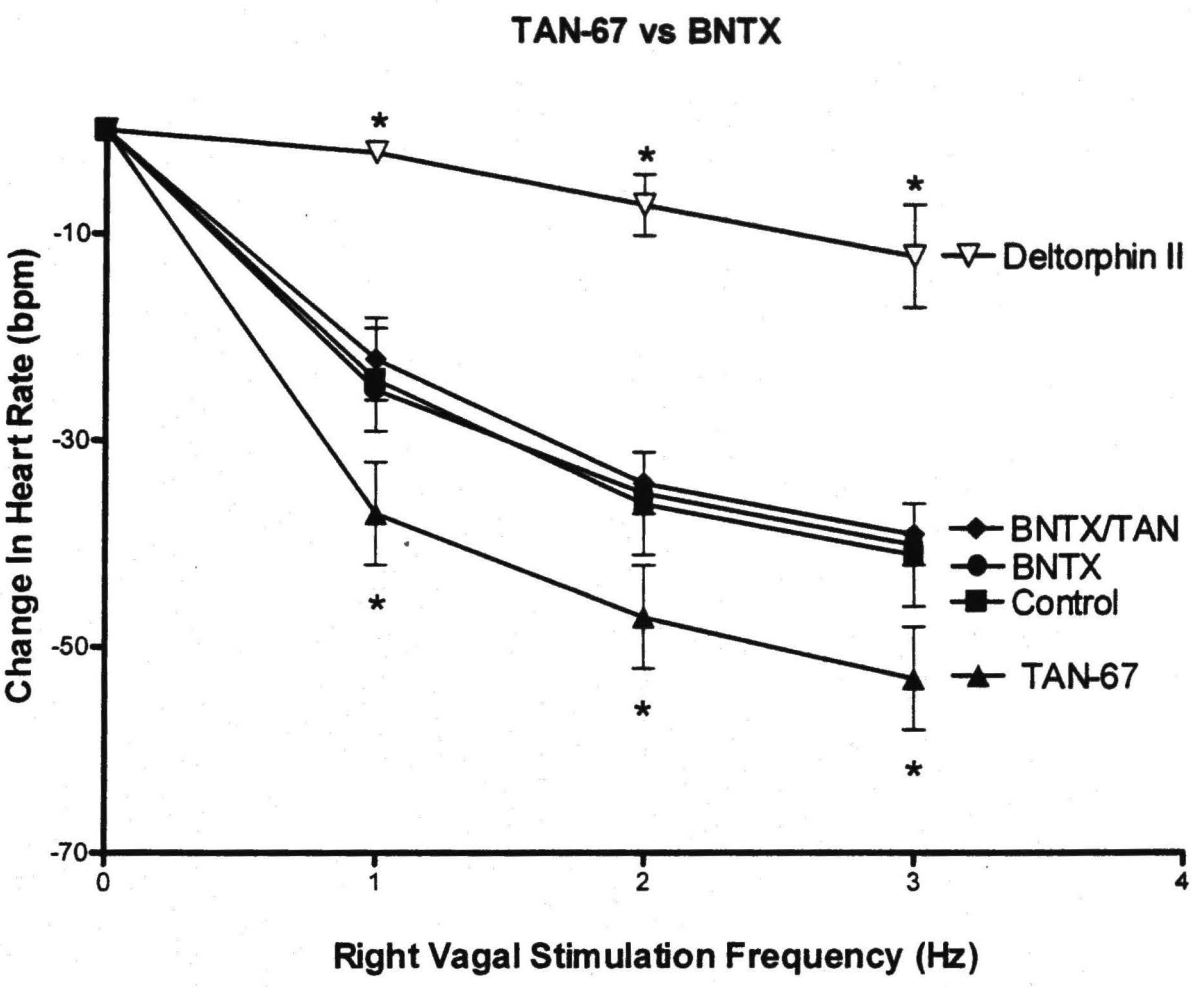
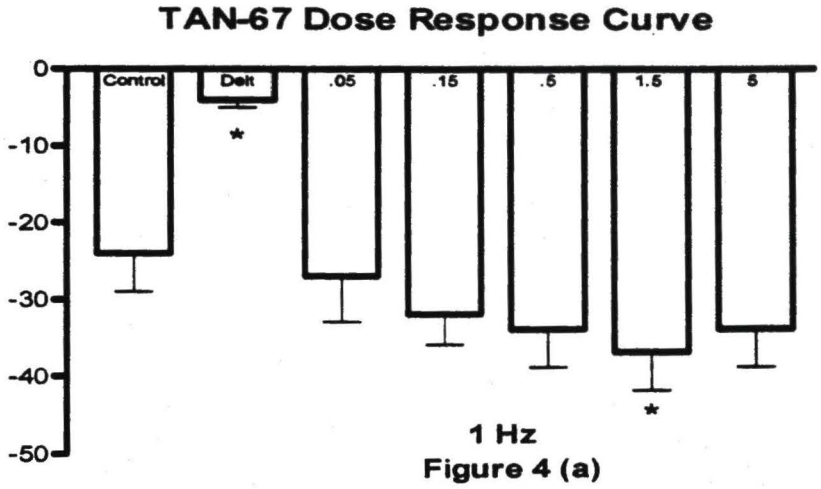
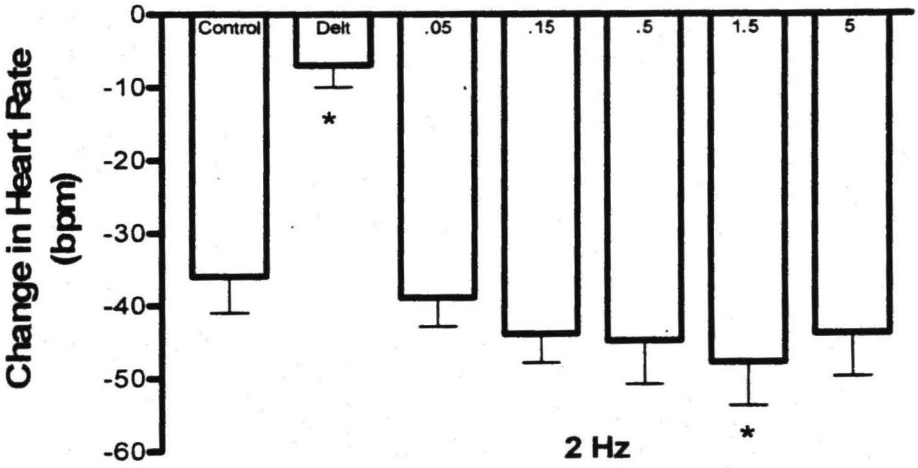

TAN-67 vs BNTX
Change In Heart Rate (bpm)
-10
-30
-50
-70
0
1
2
3
4
*
Deltorphin II
BNTX/TAN
BNTX
Control
TAN-67
Right Vagal Stimulation Frequency (Hz)

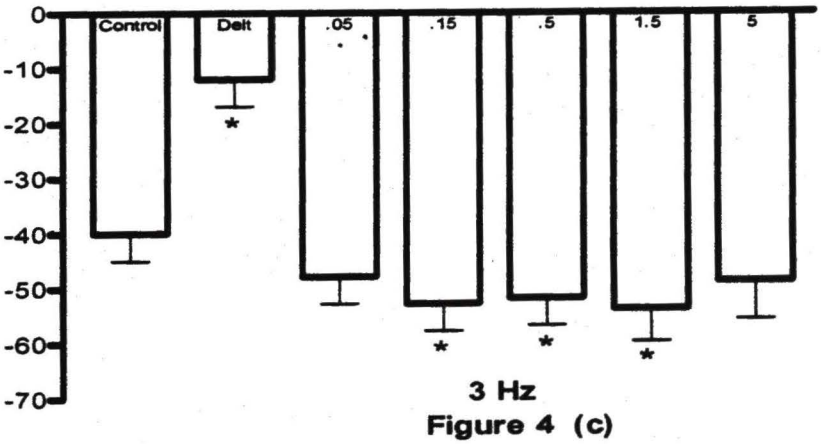
Figure 3



1 Hz
Figure 4 (a)



2 Hz
Figure 4 (b)



3 Hz
Figure 4 (c)

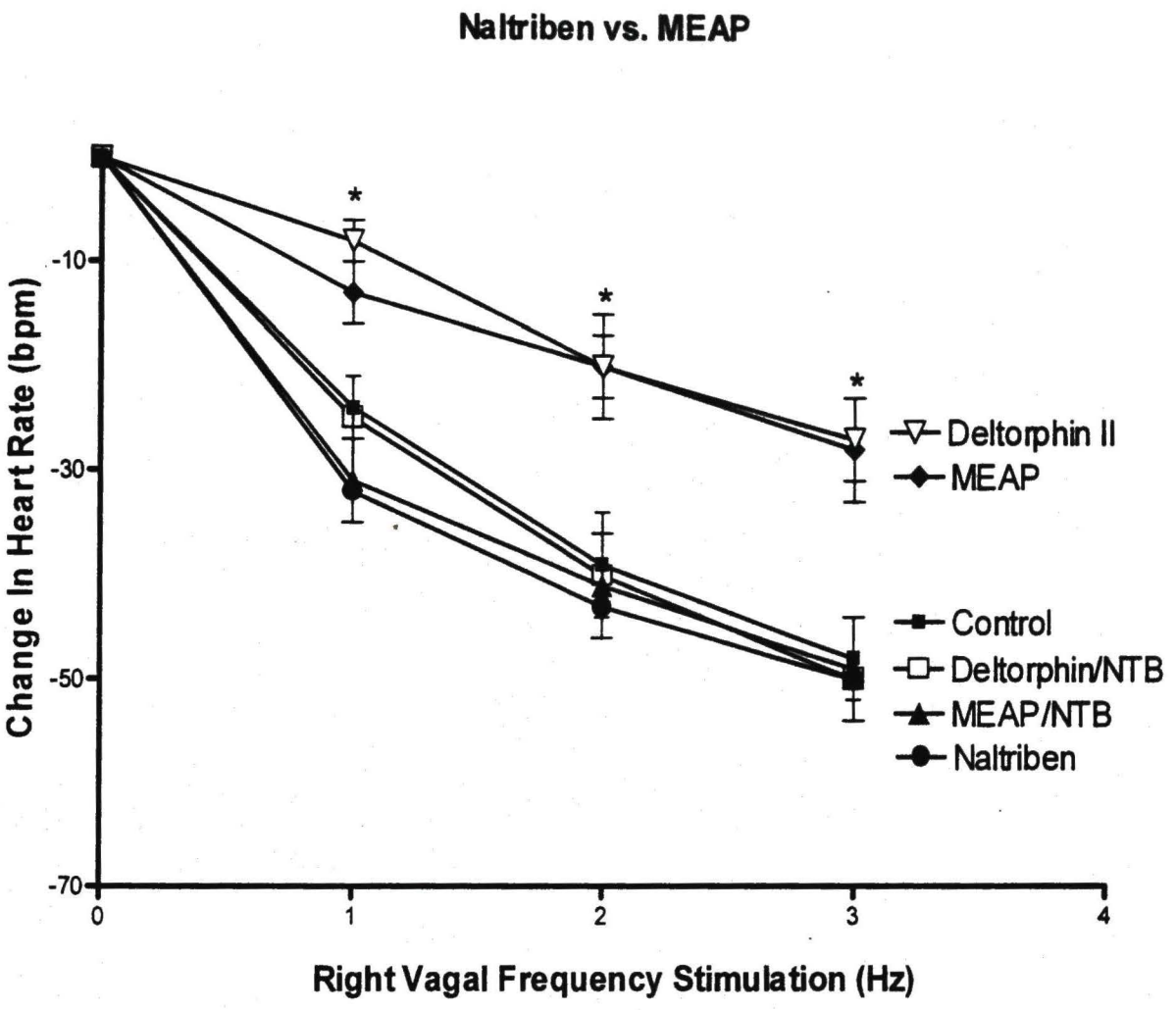
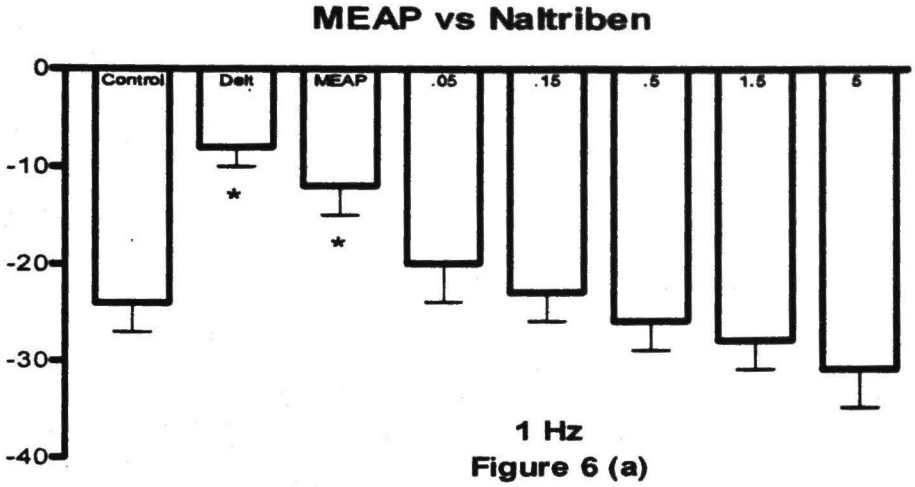
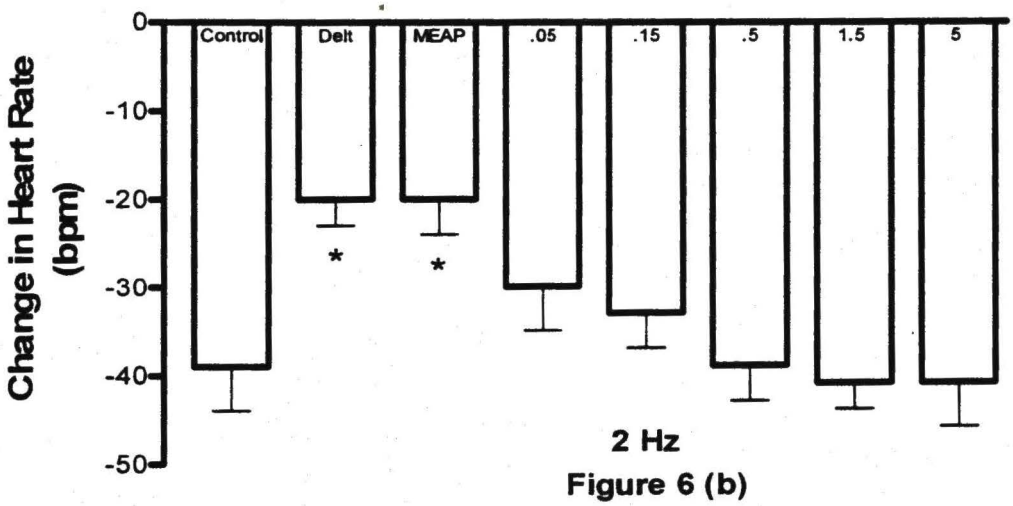

Naltriben vs. MEAP
Change In Heart Rate (bpm)
-10
-30
-50
-70
0
1
2
3
4
*
*
*
Deltorphin II
MEAP
Control
Deltorphin/NTB
MEAP/NTB
Naltriben
Right Vagal Frequency Stimulation (Hz)

Figure 5



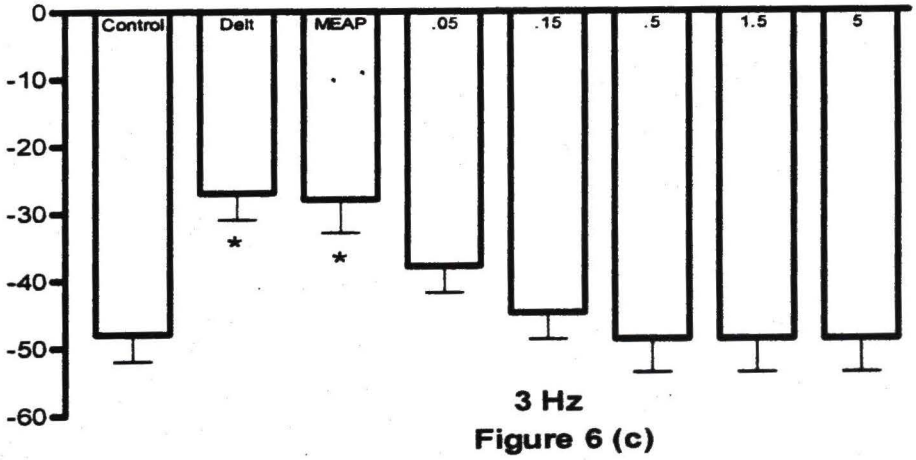
1 Hz

Figure 6 (a)



2 Hz

Figure 6 (b)



3 Hz

Figure 6 (c)

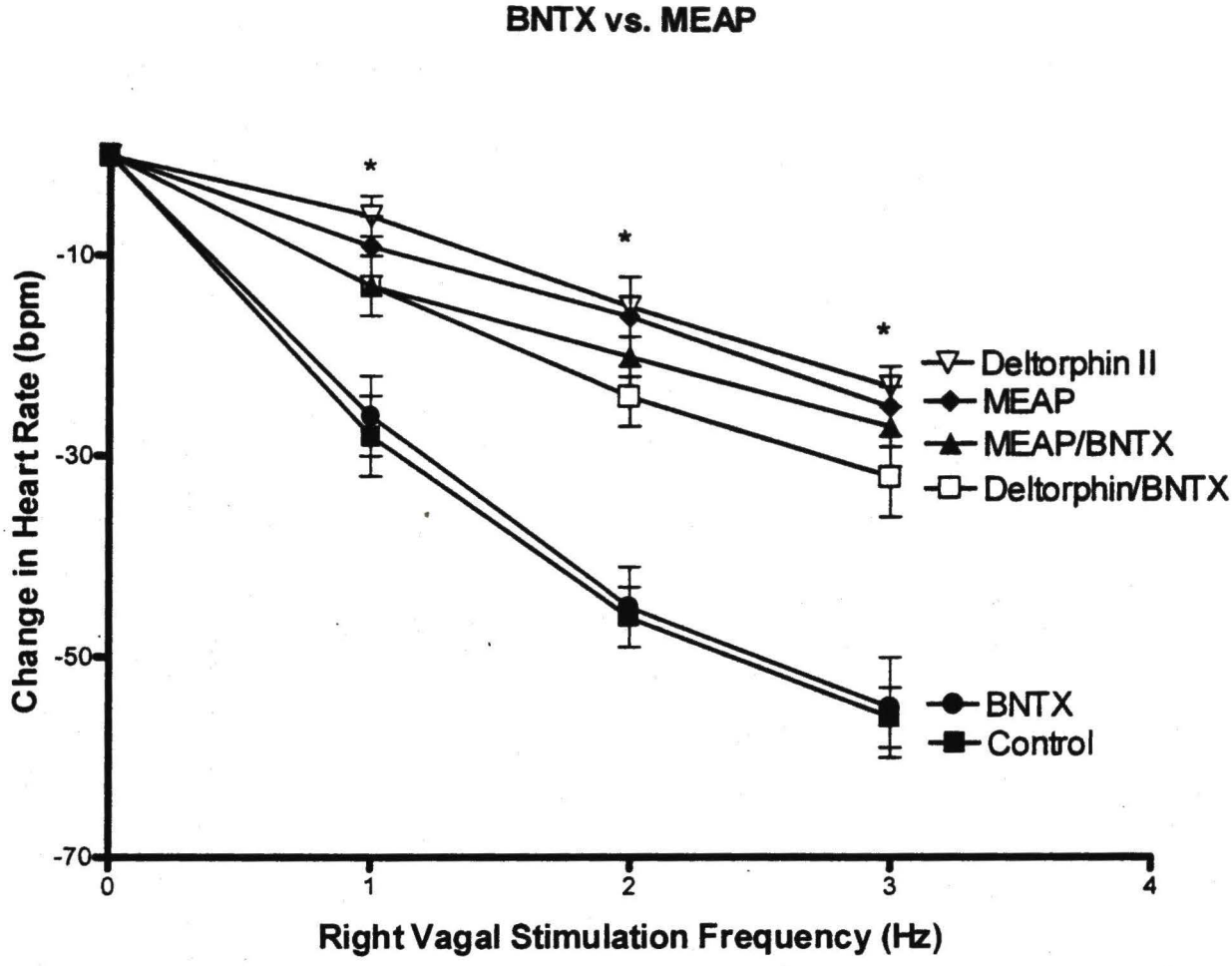
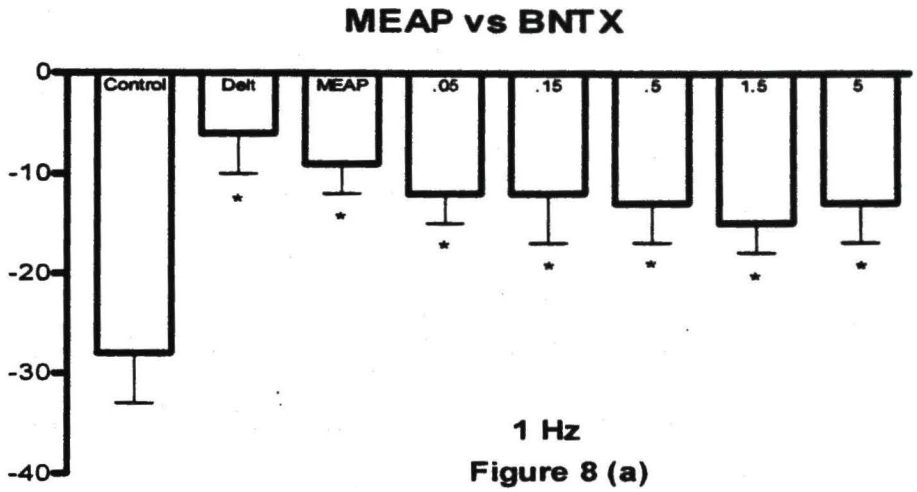
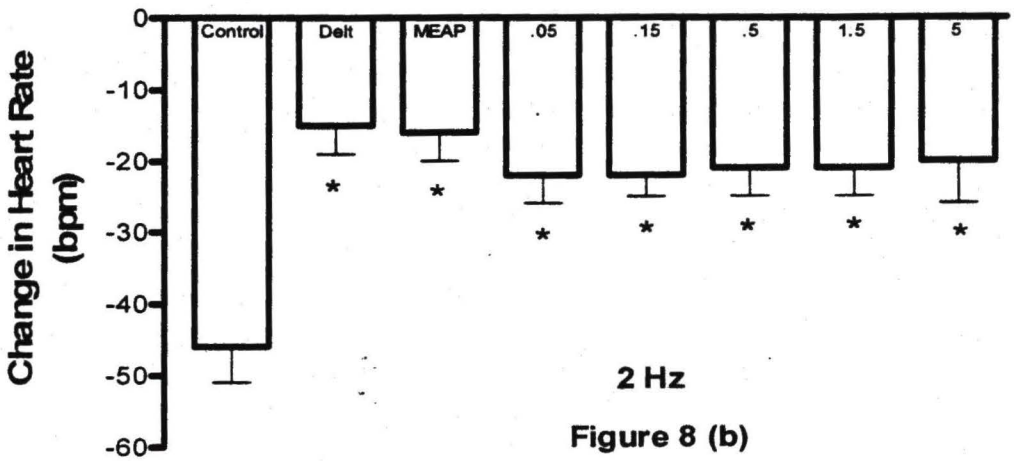

BNTX vs. MEAP
Change in Heart Rate (bpm)
-10
-30
-50
-70
0
1
2
3
4
*
*
*
Deltorphin II
MEAP
MEAP/BNTX
Deltorphin/BNTX
BNTX
Control
Right Vagal Stimulation Frequency (Hz)

Figure 7



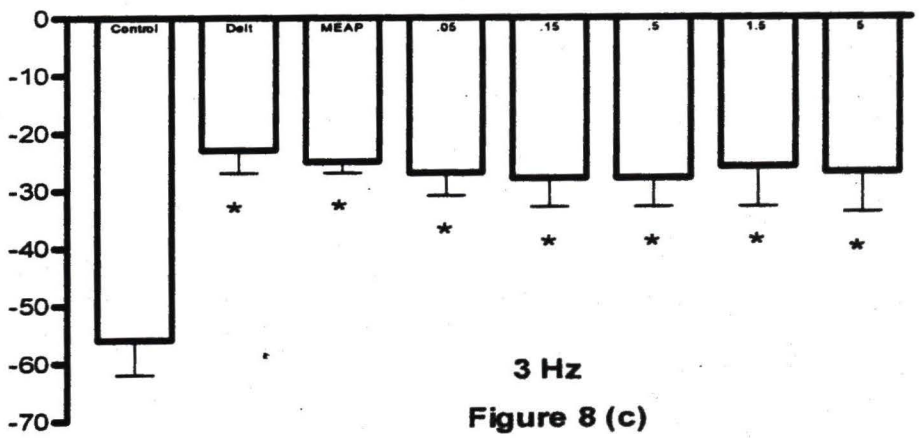
1 Hz

Figure 8 (a)



2 Hz

Figure 8 (b)



3 Hz

Figure 8 (c)

CHAPTER III

Cardiac Enkephalins Attenuate Vagal Bradycardia: interactions with the NOS1-cGMP System in the Canine Sinoatrial Node.

First Author: Farias III

Short Title: Enkephalin and Nitric Oxide Interactions in Heart Rate Control.

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 in Fort Worth
Fort Worth, Texas 76107

Address Correspondence to: James L. Caffrey
Department of Integrative Physiology
Cardiovascular Research Institute
University of North Texas Health Science Center
Fort Worth, Texas 76107
Voice (817) 735-2085
Fax (817) 735-5084
jcaffrey@hsc.unt.edu

ABSTRACT

Endogenous opioids and nitric oxide are gaining recognition as modulators of cardiac function. Enkephalins and inhibitors of nitric oxide synthase (NOS) both produce similar interruptions in the vagal control of heart rate. This study was conducted to test the hypothesis that nitric oxide 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 sinoatrial (SA) node and they were perfused with non-selective (L-NAME) 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. Introducing 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 a convergence of opioid and NO pathways was provided when the vagolytic effects of MEAP were also reversed by the addition the NO donor, SNAP, the protein kinase G activator, 8-bromocyclic-GMP, or the phosphodiesterase inhibitor, isobutyl-methyl-xanthanene. MEAP and 7-nitroindazole were individually combined with the direct acting muscarinic agonist, methacholine to evaluate potential interactions with muscarinic receptors with in the SA node. MEAP and 7-nitroindazole were unable to overcome the bradycardia produced by methacholine. These data suggest that NO and enkephalins moderate the vagal control of heart rate via interaction with converging systems that likely involve the regulation of cAMP within nodal parasympathetic nerve terminals.

INTRODUCTION

Endogenous enkephalins and nitric oxide (NO) are quickly gaining recognition for their effects on cardiac parasympathetic function. (4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15, 17, 18, 19, 20). 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 muscarinic receptors resident on the pacemaker cells (5,14). Extensive agonist/antagonist profiles indicated that the receptors responsible for these observations are δ-2-opioid receptors (15,19). These observations are consistent with the hypothesis that prejunctional δ-2-opioid receptors suppress acetylcholine release from vagal nerve terminals locally within the SA node.

Nitric oxide also appeared to moderate parasympathetic function (8, 10, 11, 13, 17, 18, 19, 23, 24). Interrupting the nitric oxide-cyclic-GMP pathway attenuated vagal bradycardia in several experimental models (8, 10, 11, 13, 17, 18, 19). NOS inhibitors reduced the negative chronotropic response to vagal stimulation in both isolated tissue and whole animal model systems (11, 12, 13, 18, 19). A selective NOS-1 (neuronal) inhibitor produced a qualitatively similar vagolytic effect suggesting that the affected enzyme was resident within the network of intrinsic and extrinsic cardiac nerves (17). In support of this thesis, NOS-1 was immunocytochemically localized in choline acetyltransferase positive cells within the atria and NOS inhibitors were ineffective when the cholinergic agonist, carbechol was substituted for direct nerve stimulation (7, 17, 21).

These findings suggested that the NO improves vagal control of heart rate by facilitating the local release of acetylcholine. None of these studies, however, clearly distinguished between actions within the node or within the nearby intra-cardiac parasympathetic ganglia.

Nitric oxide may improve vagal transmission in the heart indirectly by reducing the degradation of neuronal cyclic-AMP. The accumulating cyclic-nucleotide activates protein kinase-A, increases Ca^{+2} influx, and thus facilitates vesicular neurotransmitter release. In this proposed mechanism, NO raises cyclic-GMP by increasing guanylyl cyclase activity. Cyclic-GMP increases the activity of the cyclic-GMP dependent protein kinase, PKG which then slows the rate of cyclic-AMP hydrolysis by suppressing phosphodiesterase activity (PDE-3). This ultimately leads to an accumulation of cAMP (17, 18).

Interactions between opioids and the nitric oxide-cGMP pathway have been reported in several tissues (2, 3, 12, 22, 26). The local administration of the δ-1-selective opioid, D-Pen2, D-Pen5 (DPDPE) into the mouse brain and spinal cord decreased NOS activity (2, 3). Delta-opioids also reduced NO release in selected vascular endothelial and intestinal model systems (22, 26). 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-cyclic-GMP pathway.

This study was conducted to test the hypothesis that nitric oxide systems within the canine sinoatrial (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 anatomical location and the characteristics of the nodal NOS activity were evaluated in the SA node by microdialysis. Two strategies were employed. In the first, the vagolytic effects of NOS inhibitors and MEAP were compared with respect to time course, magnitude, specificity, and interaction with cholinergic agonists. In the second strategy, the points of interaction or convergence of NO and opioid mechanisms were tested by evaluating the ability of selected components in the NOS-cyclic-GMP pathway to override the vagolytic effect of MEAP.

METHODS

Experiments conformed to the Guide for the Care and the Use of Laboratory Animals published by the National Institutes of Health.

Surgical preparation. Forty-three mongrel dogs were anesthetized with sodium pentobarbital (32.5 mg/kg), intubated and mechanically ventilated with room air. Fluid filled catheters were inserted into the femoral artery and vein then advanced into the descending aorta and inferior vena cava, respectively. The arterial line was attached to a Statham PD23SL pressure transducer to monitor heart rate and blood pressure continuously online (Power Lab). The venous line was used to administer additional anesthetic as needed. Arterial blood gases were monitored with an blood gas analyzer (

Instrumentation Laboratories Inc.) and the pO_2 (90-120 mmHg), pH (7.35 -7.45), and pCO_2 (35-45 mmHg) were maintained in their respective physiological ranges with supplemental oxygen, bicarbonate, or by altering the minute volume, respectively.

The right and left vagus nerves were isolated through a midline surgical incision and tied off tightly with umbilical tape and the nerves were returned to the neck for later retrieval. A single dose of succinylcholine (50ug/kg) was administered intravenously to temporarily reduce involuntary muscle movements during the 10-15 minutes required for the electrosurgical incision of the right thorax and the costal-sternal cartilage for ribs 2-5. The pericardium was opened and the upper margins sutured to the body wall to provide a pericardial cradle.

Since NO reportedly also reduces norepinephrine release from cardiac sympathetic nerves, the primary cardiac sympathetic nerves were severed bilaterally (25). The sympathetic nerves were isolated as they exit the stellate ganglia, (ansa subclavia). In each case their functional identity was verified by briefly stimulating the nerve (1 Hz, 15 V, 15 sec) to observe an increase 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 positioning the probe, the line is 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} moles/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 (20). The microdialysis probe was constructed from a single one centimeter length of dialysis fiber (200 um ID, 220 um OD) and hollow silica inflow and outflow tubes (120 um ID, 170um OD). The dialysis tubing permits molecules with a molecular mass of 35,000 KD 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 analysis performed with Tukey's test for multiple comparisons. Differences determined to occur by chance with a probability <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. Non-selective (L-NAME) or NOS-1 selective (7-nitroindazole) 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 X 10^{-9} moles/min) was added to the dialysis inflow and perfused for 60 min. The right vagus nerve was stimulated for 15 sec each at 2 and 4 Hz (5 msec, 15V). One min and 45 sec were 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 one hour, each dose of each inhibitor was combined with a molar excess of the NOS substrate, L-arginine (50 x 10^{-9} moles/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-cyclic-GMP pathway in the SA node reverses the vagolytic effect of MEAP and to discern where in proposed pathway, the opioids are most likely to interact. Microdialysis probes were inserted into the SA node and after 60 min 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 intervals. MEAP (1.5×10^{-9} moles/min) was then introduced into the dialysis line perfusing the sinoatrial node and vagal stimulations were evaluated at 15 min intervals for the next 60 min. The MEAP was washed out and the restoration of baseline vagal responses was verified. Next, MEAP and L-arginine were combined and infused simultaneously into the sinoatrial node for a third hour. Vagal stimulations were conducted at 15-min intervals again as described above. Finally the combination of MEAP and L-arginine were washed out and L-arginine was infused alone for a fourth hour. In a second experimental group the inactive arginine enantiomer, D-arginine was substituted for the active substrate as a combination specificity-time control in an otherwise identical protocol.

The basic four-part protocol was also repeated in three additional independent groups of animals to explore the ability of major components of the NO-cyclic-GMP pathway to reverse or bypass the vagolytic effect of MEAP. Guanylyl cyclase, PKG and PDE were each indirectly evaluated by substituting the NO donor, SNAP, the cell permeant cyclic-GMP analogue, 8-bromo-cyclic-GMP, or the phosphodiesterase inhibitor, isobutylmethylxanthanene for L-arginine in the protocol described above.

Protocol 3: This protocol was designed to test whether the vagolytic effects of intra-nodal MEAP or the NOS-1 inhibitor resulted from direct interactions with cholinergic neurotransmitters. The microdialysis probes were positioned in the SA node

as described above. The direct acting muscarinic agonist, methacholine (1 x 10^{-7} mole/min) was introduced into the SA node by dialysis for 30 minutes to establish a consistent bradycardia equivalent to that obtained during a submaximal vagal stimulation. Heart rate was recorded at 5-min intervals and after 30 min the methacholine was washed out. When heart rate returned to baseline, the right vagus nerve was stimulated for 15 sec (3 Hz) and the decline in heart rate was recorded. MEAP (5 x 10^{-9} moles/min) was introduced into the dialysis inflow and perfused for five min. After 5 min, the vagus was retested to verify that the vagolytic effect of intranodal MEAP was intact these animals. MEAP was washed out and methacholine (1 x 10^{-7} mole/min) and MEAP (5 x 10^{-9} moles/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 with 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. Though 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 sinoatrial node via cardiac microdialysis, regardless of dose.

L-NAME dose response (n=5). The purpose of this portion of protocol 1 was to determine if 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-hr experimental protocol. When L-NAME (5×10^{-9} moles/min) was introduced into the nodal interstitium, vagally mediated bradycardia gradually declined by 50% with the maximal effect illustrated in the upper line in figure 1. 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} moles/min). As indicated among the lower curves in figure 1, L-arginine restored vagally mediated bradycardia to values not different from those observed during vehicle administration.

Figure 2 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} moles/min (center panels) 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 (left panels) and no additional effect was observed at three times the dose (right panels). The effect did appear to develop somewhat earlier at the highest dose, suggesting that diffusion to the target may have contributed 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's 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 to enhance vagal bradycardia suggested that the endogenous NOS substrate was sufficient under the current experimental conditions.

7-Nitroindazole dose response (n=5). 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-hr protocol. The NOS-1 selective inhibitor, 7-nitroindazole (5×10^{-9} moles/min) gradually reduced vagal transmission within the SA node by a maximum of 35% (figure 3, upper line). An excess of the NOS substrate, L-arginine prevented the 7-nitroindazole-mediated reduction in vagal transmission (figure 3, lower curves) when combined in the dialysis inflow. This provided support for NOS participation in the response to 7-nitroindazole (fig 1).

Figure 4 illustrates the results of the entire dose response and the temporal development of the effect for comparison with L-NAME above. The maximal effect is evident at 5×10^{-9} moles/min (center panels) and appears to reach that maximum between 15 and 30 min of exposure. The effect is no greater at three times the dose (right panels) and completely absent at one-tenth the dose (left panels). The full inhibition does appear 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 x 10^{-8} moles/min) and 7-nitroindazole for one hour completely prevented the 7-nitroindazole-mediated reduction in vagal bradycardia regardless of the 7-nitroindazole dose employed. To conserve space only the one hour values for the L-arginine/7-nitroindazole combination are illustrated as the last bar in each panel. L-arginine did not appear to increase the vagally mediated decline in heart rate compared to that observed during vehicle administration suggesting again that the endogenous substrate available was not limiting during these experimental conditions.

L-arginine vs MEAP (n=5). The purpose of this protocol was to test whether enhancing selected components of the NOS-cyclic-GMP pathway could reverse or bypass the vagolytic effect of MEAP. The upper curve in figure 5 illustrates the vagolytic effect of adding MEAP into the SA node interstitium after 60 min exposure. The dose of MEAP (5 x 10^{-9} moles/min) represents the ED100 as determined in prior studies (20). This dose routinely produces a 65-75% inhibition of vagal bradycardia as indicated in figure 5. The combined administration of L-arginine and MEAP reversed the vagolytic effect of MEAP as illustrated among the lower curves in figure 5. The vagal bradycardia during the administration of L-arginine (5 x 10^{-8} moles/min) alone was likewise not different from the vagally-mediated bradycardia observed during vehicle administration.

Figure 6 depicts the development of each response during each one-hour portion of the protocol. The left panel illustrates the effect of repeated vagal frequency responses

during one hour of vehicle administration. No attrition or enhancement was evident. The addition of MEAP to the dialysis inflow reduced the vagally-mediated bradycardia by approximately 75% at the first evaluation 15 min later (left center panels). 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 washing out the MEAP/L-arginine combination, 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 cyclic-GMP 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 non-substrate, D-arginine was substituted for L-arginine in the same protocol to evaluate potential non-specific 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 (figure 7,

upper curves) and D-arginine alone had no effect on the vagally mediated decline in heart rate. The complete temporal pattern for D-arginine (Figure 8) 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 Figure 6 was not the consequence of a slowly developing tachyphylaxis.

SNAP vs MEAP (n=5). 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. MEAP produced a typical vagolytic response as illustrated for 60 min in Figure 9 (upper curve). The vagal bradycardia was restored to values indistinguishable from those obtained during control when SNAP and MEAP were combined (lower curves) 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 (Figure 10. right center panels). 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 vs MEAP (n=5). 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 cyclic-GMP. In these studies, the cell permeant, cyclic-GMP analogue, 8-bromo-cyclic-GMP was substituted for L-Arginine in the original

protocol. Once again MEAP produced a clear vagolytic response as illustrated for 60 min in Figure 11 (upper curve). Much like L-arginine and SNAP, the cyclic-GMP analogue completely reversed the vagolytic effect of MEAP when 8-bromo-cyclic-GMP 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 (Figure 12). The cyclic-GMP analogue did not alter the baseline vagal response when administered alone suggesting again that flux through this pathway is adequate under control conditions.

Isobutyl methyl xanthanene vs MEAP (n=5). The purpose of this protocol was to test whether the ability of 8-bromo-cyclic-GMP to reverse the vagolytic effect of MEAP was consistent with the proposal that cyclic-GMP raises cyclic-AMP by reducing phosphodiesterase activity. In these experiments the phosphodiesterase inhibitor, isobutyl methyl xanthanene 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 (Figure 13, upper curve). These vagolytic effects of MEAP were reversed by the addition of the phosphodiesterase inhibitor and the heart rate response to vagal stimulation was restored to that observed during vehicle administration (lower curves). This observation suggested that an increased phosphodiesterase activity contributed to the vagolytic activity of MEAP. The control responses during vehicle administration and the vagolytic effects of MEAP were each consistent respectively through the first and second hours of the protocol (Figure 14, right panels). In contrast to SNAP and 8-bromo-cyclic-GMP, 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 vs MEAP (n=5). MEAP was 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 (Figure 15, upper panel). After washing out the methacholine, the right vagus was stimulated at 3 Hz producing sharp 15 sec decline in heart rate (lower panel, right side). MEAP was introduced into the dialysis line as a positive control. MEAP did not alter the resting baseline heart rate. After 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 not supramaximal in these animals (lower panel, left side). 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 methacholine alone. This observation suggests there was no interaction between MEAP and nodal muscarinic receptors.

Methacholine vs 7-nitroindazole (N=5). The NOS-1 selective inhibitor, 7-nitroindazole 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 (Figure 16, upper panel) similar to that observed in the prior study. After washing out the methacholine, the right vagus was stimulated at 3 Hz producing brief 15 sec 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 5 min exposure, the MEAP produced a clear interruption in vagal transmission (Figure 16 lower panel, right side). Methacholine and 7-nitroindazole 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 methacholine alone. 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. However, the comparison of the quantitative and temporal dynamics of vagal inhibition by MEAP and by NOS inhibitors suggested that

MEAP and the NOS-cGMP pathway converged on the same end component independently, to modulate vagal bradycardia.

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 were less effective at attenuating vagal bradycardia than MEAP. These agents attenuated vagal bradycardia by 35-50 percent while MEAP produced a 60-70 percent inhibition. Temporally, the vagolytic effects of MEAP developed and resolved respectively within minutes of initiating or discontinuing exposure (5). This time frame contrasts with the 45-60 minutes needed for both NOS inhibitors to take effect. Reversal of the enkephalin mediated vagolysis by opioid antagonists was also faster compared to 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 phosphodiestrase in the restoration of vagal function during exposure to MEAP. The finding that the phosphodiesterase inhibitor, IBMX reversed the enkephalin-mediated vagolysis suggested that MEAP interrupted vagal transmission by suppressing adenylate cyclase and lowering cyclic-AMP within the vagal nerve terminals. As neuromodulators, opioids are widely recognized for their ability to suppress neurotransmitter release through the Gi/Go-coupled inhibition of adenylate cyclase activity. Since the NOS-cGMP pathway promotes vagal transmission by suppressing phosphodiesterase activity, the convergence of enkephalin and NO at cyclic-AMP provides an attractive explanation of the current findings.

Proposed mechanisms for cholinergic neurotransmission include the activation of Gs-coupled, cyclic-AMP dependent, second messenger systems (19). In this mechanism adenylate cyclase activity increases cyclic-AMP within prejunctional terminals and activates protein kinase A (PKA). 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 (18, 19). Since neuronal opioid receptors interact with inhibitory G-proteins that suppress adenylyl cyclase (24), the resulting decrease in cyclic-AMP would provide a logical explanation of the vagolytic effect of MEAP (24). In contrast, NO promotes vagal transmission by increasing neuronal cyclic-AMP indirectly through a reduction in

the rate of cyclic nucleotide hydrolysis (17, 18). When neuronal NOS is activated in vagal nerve terminals, the NO produced activates guanylyl cyclase and increases the concentration of cyclic-GMP. Once formed cyclic-GMP activates PKG which in turn inactivates phosphodiesterase 3 (PDE3). PDE3 normally hydrolyzes cyclic-AMP to 5' AMP and thus inhibition of PDE3 leads to the accumulation of cAMP. As stated earlier, an increase in cyclic-AMP should facilitate vagal transmission and promote vagal bradycardia (17, 18). The magnitude and temporal character of the facilitation would however depend upon the existing cyclase activity and ambient concentration of cyclic-AMP. As such, the NO pathway may determine the responsiveness of vagal transmission to other neuromodulators that increase or decrease adenylate cyclase activity directly.

The Interaction between MEAP and the NOS-cGMP Pathway is localized prejunctionally in the 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 have carefully localized the vagolytic effect of MEAP within the SA node (14). 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 current findings are consistent with reports that have localized nNOS in vagal nerve terminals innervating the mouse and rat sinoatrial node

and the guinea pig atria (7,22). Neither MEAP or the NOS inhibitors appear to interact with cardiac pacemakers since all three agents were ineffective when the bradycardia was produced by the intra-nodal 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, carbamylcholine (18). Although that guinea pig model does not distinguish between potential interactions at ganglionic and pacemaker junctions, the data are consistent with the current 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 "nNOS" 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 to its non-selective analogue, L-NAME (35% vs 50%). If this difference is real, more than one NOS isoform, perhaps eNOS may have contributed to the overall response. However, immunocytochemical approaches failed to demonstrate eNOS in vagal nerve terminals in the sinoatrial node (7, 22) 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 PDE3 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.

Physiological Significance.

Both MEAP and Nitric oxide 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 PDE3. Thus by regulating of 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 NOS to restore cAMP and vagal transmission slowly during exposure to MEAP since 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 limiting under control conditions since 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 also appears to be rate limiting since 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 NOS activity in atrial parasympathetics (18). Furthermore, pathologic states such as hypertension and congestive heart failure are both associated with impaired NO production (16, 25) and impaired vagal control of the heart.

Conclusions: 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, cAMP. Both interactions with vagal transmission are likely prejunctional, presumably mediated within parasympathetic nerve terminals.

REFERENCES

1. **Alderton WK, Cooper CE, and Knowles RG**. Nitric oxide synthases, structure, function and inhibition. *Biochem J* 357: 593-615, 2001

2. **Bhargava HN, and Cao YJ**. Effect of chronic administration of [D-Pen2, D-Pen5] enkephalin on the activity of nitric oxide synthase in brain regions and spinal cord of mice. *Peptides* 19: 113-7, 1998.

3. **Bhargava HN, and Cao YJ**. Effect of chronic administration of morphine, U-50, 488H and [D-Pen2, D-Pen5] enkephalin on the concentration of cGMP in brain regions and spinal cord of mice. *Peptides* 18: 1629-1634, 1997.

4. **Caffrey JL**. Enkephalin inhibits vagal control of heart rate, contractile force, and coronary blood flow in canine heart in vivo. *J. Autonomic Nervous Sys* 76:75-82, 1999.

5. **Caffrey JL, Mateo Z, Napier LD, Gaugle JF, and Barron BA**. Intrinsic cardiac enkephalins inhibit vagal bradycardia in the dog. *Am. J. Physiol.* 268 (Heart Circ Physiol. 37): H848-H855, 1995.

6. **Caffrey JL, Wooldridge CB, and Gaugl JF**. The interaction of endogenous opiates with autonomic circulatory control in the dog. *Circ. Shock*, 17: 233-242, 1985.

7. **Choate JK, Danson EJF, Morris JF, and Paterson DJ**. Peripheral vagal control of heart rate is impaired in neuronal NOS knockout mice. *Am J Physiol (Heart and Circ Physiol)*, 281: H2310-H2317, 2001

8. **Chowdhary S, Vaile JC, Fletcher J, Ross HF, Coote JH, and Townend JN**. Nitric oxide and cardiac autonomic control in humans. *Hypertension* 36:264-269, 2000.

9. **Chowdhary S, and Townend JN**. Role of nitric oxide in the regulation of cardiovascular autonomic control. *Clin. Sci.* 97:5-17, 1999.

10. **Conlon K, and Kidd C**. N euronal N itric o xide facilitates vagal chronotropic and dromotropic actions on the heart. J. *Auton. Nerv. Sys*. 75:136-146, 1999.

11. **Conlon K, Collins T, and Kidd C**. The role of nitric oxide in the control by the vagal nerves of the heart of the ferret. *Exp. Physiol* 83: 469-480, 1998.

12. **Deliconstantinos G, Villiotou V, and Stavrides JC**. Met- enkephalin receptor mediated increase of membrane fluidity modulates nitric oxide and cGMP production in rat brain synaptosomes. *Neurochem. Res*. 20: 217-224, 1995.

13. **Elvan A, Rubart M, and Zipes DP**. NO modulates autonomic effects on sinus discharge rate and AV nodal conduction in open chest dogs. *Am. J. Physiol.*272 (*Heart Circ. Physiol.* 41): H263-H271, 1997.

14. **Farias M, Jackson K, Stanfill A, and Caffrey JL**. Local opiate receptors in the sinoatrial node moderate vagal bradycardia. *Auto. Neuro.* 87:9-15, 2001.

15. **Farias M, Jacskon K, Darice Yoshishige, and Caffrey JL**. Cardiac Enkephalins Interrupt Vagal Bradycardia Via δ-2 Opioid Receptors in the Sinoatrial Node. *Am J Physiol (Heart and Circ Physiol)* In press, 2002.

16. **Hare JM, and Colucci WS.** Role of nitric oxide in the regulation of myocardial function. *Prog. Cardiovasc. Dis.* 38 (2): 155-66,1995.

17. **Herring N, Golding S, and Paterson DJ**. Pre-synaptic NO-cGMP pathway modulates vagal control of heart rate in isolated adult guinea pig atria. *J. Mol. Cell Cardiol.* 32: 1795-1804, 2000.

18. **Herring N, Danson EJF, and Paterson DJ.** Cholinergic control of heart rate by nitric oxide is site specific. News Physiol Sci17:202-206,2002.

19. **Israel M, and Manaranche R.** The release of acetylcholine: from a cellular towards a molecular mechanism. *Biol Cell*, 55: 1-14. 1985.

20. **Jackson K, Farias M, Stanfill A, and Caffrey JL.** Delta opiod receptors inhibit vagal bradycardia in the sinoatrial node. *J. Cardiovasc. Pharmacol.* In Press, 2001.

21. **Jackson K, Farias M, Stanfill A, and Caffrey JL.** Transient arterial occlusions raises enkephalin in the sinoatrial node and improves vagal bradycardia in canines. *Auton. Neuro.* In Press, 2001.

22. **Klimaschewski L, Kummer W, Mayer B, Courand JY, Preissler U, Philippin B, and Heym C.** Nitric oxide synthase in cardiac nerve fibers and neurones of rat and guinea pig heart. *Circ. Res.* 71: 1533-1537, 1992.

23. **Lenard L, Halmai V, and Bartho L.** Morphine contracts the Guinea pig ileal circular muscle by interfering with a nitric oxide mediated tonic inhibition. *Digestion* 60: 562-6, 1999.

24. **Salto C, Calvet R, Guitart X, Solsona C and Marsal J.** Opiates depress ACh and ATP release from cholinergic synaptosomes by blocking calcium uptake. *Toxicol Appl Pharmacol* 106 (1):20-7, 1990

25. **Searles CD**. The nitric oxide pathway and oxidative stress in heart failure. Congest Heart Fail 8 (3): 142-7, 2002.

26. **Sears CE, Choate JK, and Paterson DJ**. NO-cGMP pathway accentuates the decrease in heart rate caused by cardiac vagal nerve stimulation. *J. Appl. Physiol.* 86: 510-516, 1999.

27. **Schwarz P, Diem R, Dun NJ, and Fosterman U.** Endogenous and exogenous nitric oxide inhibits norepinephrine release from rat heart sympathetic nerves . *Circ Res*, 77: 841-848, 1995.

28. **Stefano GB, Salzet M, Hughes TK, and Bilfinger TV**. Delta II opioid receptor subtype on human vascular endothelium uncouples morphine stimulated nitric oxide release. *Int. J. Cardiol.* 30: 64 suppl 1: S43-51, 1998.

29. **Tanaka K, Okshima H, Hiroyasu E, and Tnemichi E**. Direct synaptic contacts of nitric oxide synthase-immunoreactive nerve terminals on the neurones of the intracardiac ganglia of the guinea pig. *Neurosci. Lett.* 158: 67-70, 1993.

30. **Younes A, Pepe S, Barron BA, Spurgeon H, Lakatta EG, and Caffrey JL**. High rates of proenkephalin processing in rat heart. *Am J Physiol* 279: H1989- H1998, 2000.

LEGEND

Figure 1: This graph represents the heart rate/frequency response during right vagal nerve stimulation during the nodal delivery of saline vehicle, L-NAME (5 x 10^{-9} moles/min, 60 min) alone and combined with L-arginine (50 x 10^{-9} moles/min, 60 min). * = Significantly different from control, P< .05.

Figure 2: This graph represents the heart rate/frequency response during right vagal nerve stimulations with increasing doses of L-NAME and with each dose combined with L-arginine (L-arg, 50 x 10^{-9} moles/min, 60 min). In an effort to save space, only the 60 min value for L-arginine treatment is shown. Vagal stimulations were performed at 15 minute intervals during the 60 min treatments. * = Significantly different from control, P< .05.

Figure 3: This graph represents the heart rate/ frequency response during right vagal nerve stimulation during the nodal delivery of saline vehicle, 7-nitroindazole (7 Nitro, 5 x 10^{-9} moles/min, 60 min) alone and combined with L-arginine (L-arg, 50 x 10^{-9} moles/min, 60 min). * = Significantly different from control, P< .05.

Figure 4: This graph represents the heart rate/ frequency response during right vagal nerve stimulations with increasing doses of 7-nitroindazole and with each dose combined with L-arginine (L-arg, 50 x 10^{-9} moles/min, 60 min). In an effort to save space, only the 60 min value for L-arginine treatment is shown . Vagal stimulations were

performed at 15 minute intervals during the 60 min treatments. * = Significantly different from control, P< .05.

Figure 5: This graph represents the heart rate/frequency response to right vagal nerve stimulation during the nodal delivery of saline vehicle, L-arginine alone (5 x 10^{-8} moles/min , 60 min), MEAP (1.5 x 10^{-9} moles/min, 60 min) alone and combined with L-arginine (5 x 10^{-8} moles/min). * = Significantly different from control, P< .05.

Figure 6: This graph represents the heart rate/frequency response during right vagal nerve stimulation for the entire 60 minute time course during saline vehicle, MEAP, L-arginine and MEAP combined with L-arginine. Concentrations are the same as figure 5. Vagal stimulations were performed at 15 minute intervals. * = Significantly different from control, P< .05.

Figure 7: This graph represents the heart rate/frequency response to right vagal nerve stimulation during the nodal delivery of saline vehicle, D-arginine alone (5 x 10^{-8} moles/min, 60 min), MEAP (1.5 x 10^{-9} moles/min, 60 min) alone and combined with D-arginine (5 x 10^{-8} moles/min moles/min,). * = Significantly different from control, P< .05.

Figure 8: This graph represents the heart rate/frequency response during right vagal nerve stimulation for the entire 60 minute time course during saline vehicle, MEAP, D-arginine

and MEAP combined with D-arginine. Concentrations for each treatment are the same as in figure 7. Vagal stimulations were performed at 15 minute intervals. * = Significantly different from control, P< .05.

Figure 9: This graph represents the heart rate/frequency response to right vagal nerve stimulation during the nodal delivery of saline vehicle, SNAP alone (5×10^{-8} moles/min, 60 min), MEAP (1.5×10^{-9} moles/min, 60 min) alone and combined with SNAP (5×10^{-8} moles/min). * = Significantly different from control, P< .05.

Figure 10: This graph represents the heart rate/frequency response during right vagal nerve stimulation for the entire 60 minute time course during saline vehicle, MEAP, SNAP and MEAP combined with SNAP. Concentrations for each treatment are the same as in figure 9. Vagal stimulations were performed at 15 minute intervals. * = Significantly different from control, P< .05.

Figure 11: This graph represents the heart rate/frequency response to right vagal nerve stimulation during the nodal delivery of saline vehicle, 8-bromo-cGMP alone (5×10^{-8} moles/min, 60 min), MEAP (1.5×10^{-9} moles/min, 60 min) alone and combined with 8-bromo-cGMP (5×10^{-8} moles/min). * = Significantly different from control, P< .05.

Figure 12: This graph represents the heart rate/frequency response during right vagal nerve stimulation for the entire 60 minute time course during saline vehicle, MEAP, 8-

bromo-cGMP, and MEAP combined with 8-bromo-cGMP. Concentrations for each treatment are the same as in figure 11. Vagal stimulations were performed at 15 minute intervals. * = Significantly different from control, P< .05.

Figure 13: This graph represents the heart rate/frequency response to right vagal nerve stimulation during the nodal delivery of saline vehicle, IBMX alone (5×10^{-8} moles/min, 60 min), MEAP (1.5×10^{-9} moles/min, 60 min) alone and combined with IBMX (5×10^{-8} moles/min). * = Significantly different from control, P< .05.

Figure 14: This graph represents the heart rate/frequency response during right vagal nerve stimulation for the entire 60 minute time course during saline vehicle, MEAP, IBMX, and MEAP combined with IBMX. Concentrations for each treatment are the same as in figure 13. Vagal stimulations were performed at 15 minute intervals. * = Significantly different from control, P< .05.

Figure 15: This graph represents the change in heart rate produced by 30 minute nodal delivery of the direct acting muscarinic agonist , methacholine (meth, 1×10^{-7} mole/min) alone and combined with MEAP (1.5×10^{-9} moles/min). The lower panel compares 3 Hz vagal stimulations during vehicle infusion and nodal delivery of MEAP (1.5×10^{-9} moles/min) with the 30 min values from the upper panel. * = Significantly different from control, P< .05.

Figure 16: This graph represents the change in heart rate produced by 30 minute nodal delivery of the direct acting muscarinic agonist , methacholine (meth, 1 x 10^{-7} mole/min) alone and combined with 7-nitroindazole (1.5 x 10^{-9} moles/min). The lower panel compares 3 Hz vagal stimulations during vehicle infusion and nodal delivery of MEAP (1. 5 x 10^{-9} moles/min) with the 30 min values from the upper panel. * = Significantly different from control, $P < .05$.

Table 1. Baseline Hemodynamic Variables

Groups	Control		Treatment		Washout	
	HR	MAP	HR	MAP	HR	MAP
MEAP	100±8	98±9	120±7	113±7	129±7	119±9
L-NAME	98±6	100±5	115±6	102±8	124±6	115±6
7-NT	117±5	116±7	118±8	116±6	119±5	120±7
L-arg	115±4	112±7	114±6	116±9	118±6	117±5
SNAP	129±9	108±5	131±8	113±9	129±5	119±7
cGMP	117±7	101±7	119±6	102±5	117±6	104±6
D-arg	112±9	108±5	115±8	106±9	116±8	109±7
IBMX	116±7	112±7	113±6	114±5	115±6	115±5

L-NAME Attenuates Vagal Bradycardia

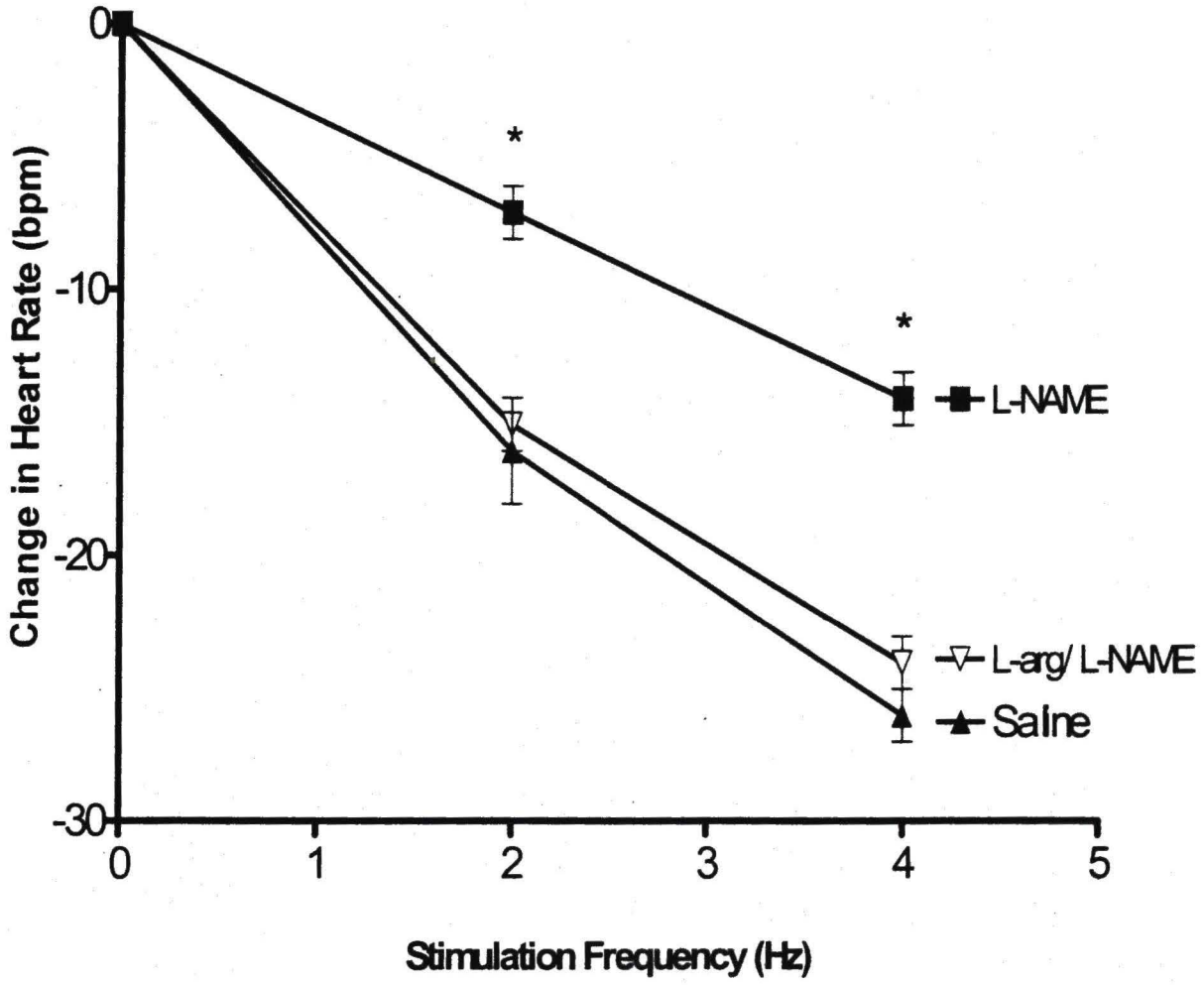
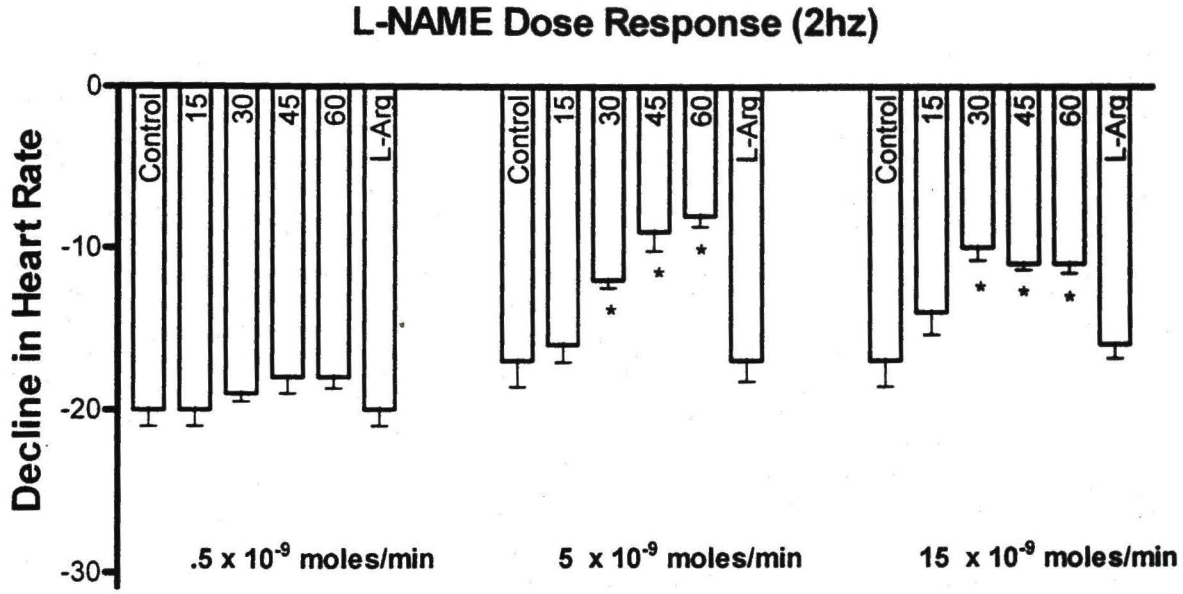


Figure 1


L-NAME Dose Response (2hz)
Decline in Heart Rate
0
-10
-20
-30
Control
15
30
45
60
L-Arg
.5 x 10-9 moles/min
5 x 10-9 moles/min
15 x 10-9 moles/min

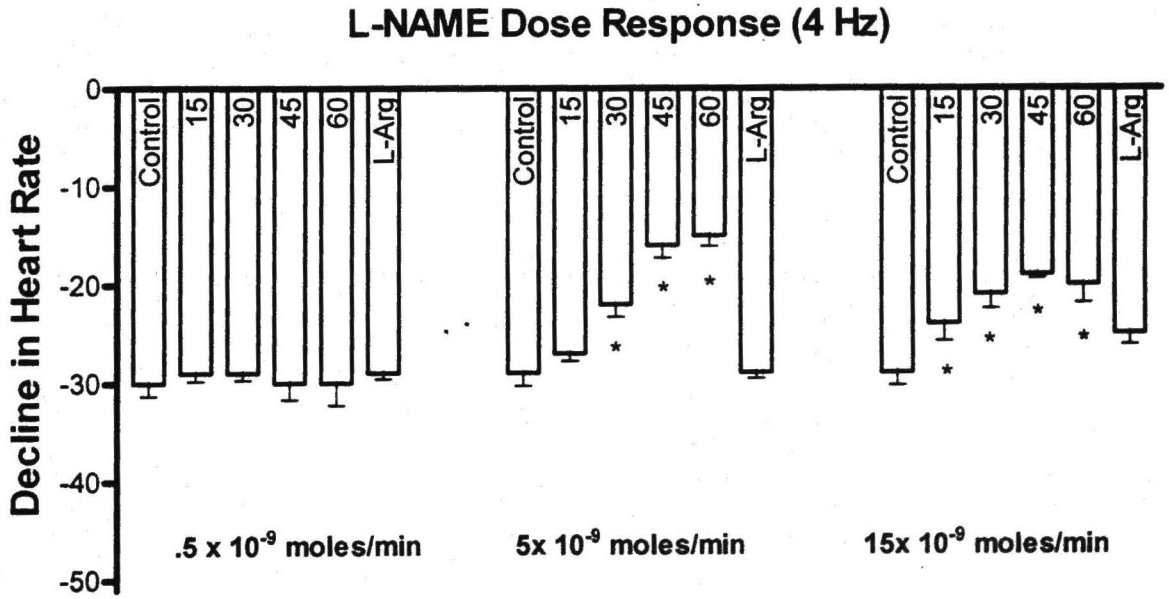

L-NAME Dose Response (4 Hz)
Decline in Heart Rate
0
-10
-20
-30
-40
-50
Control
15
30
45
60
L-Arg
.5 x 10-9 moles/min
5x 10-9 moles/min
15x 10-9 moles/min

Figure 2

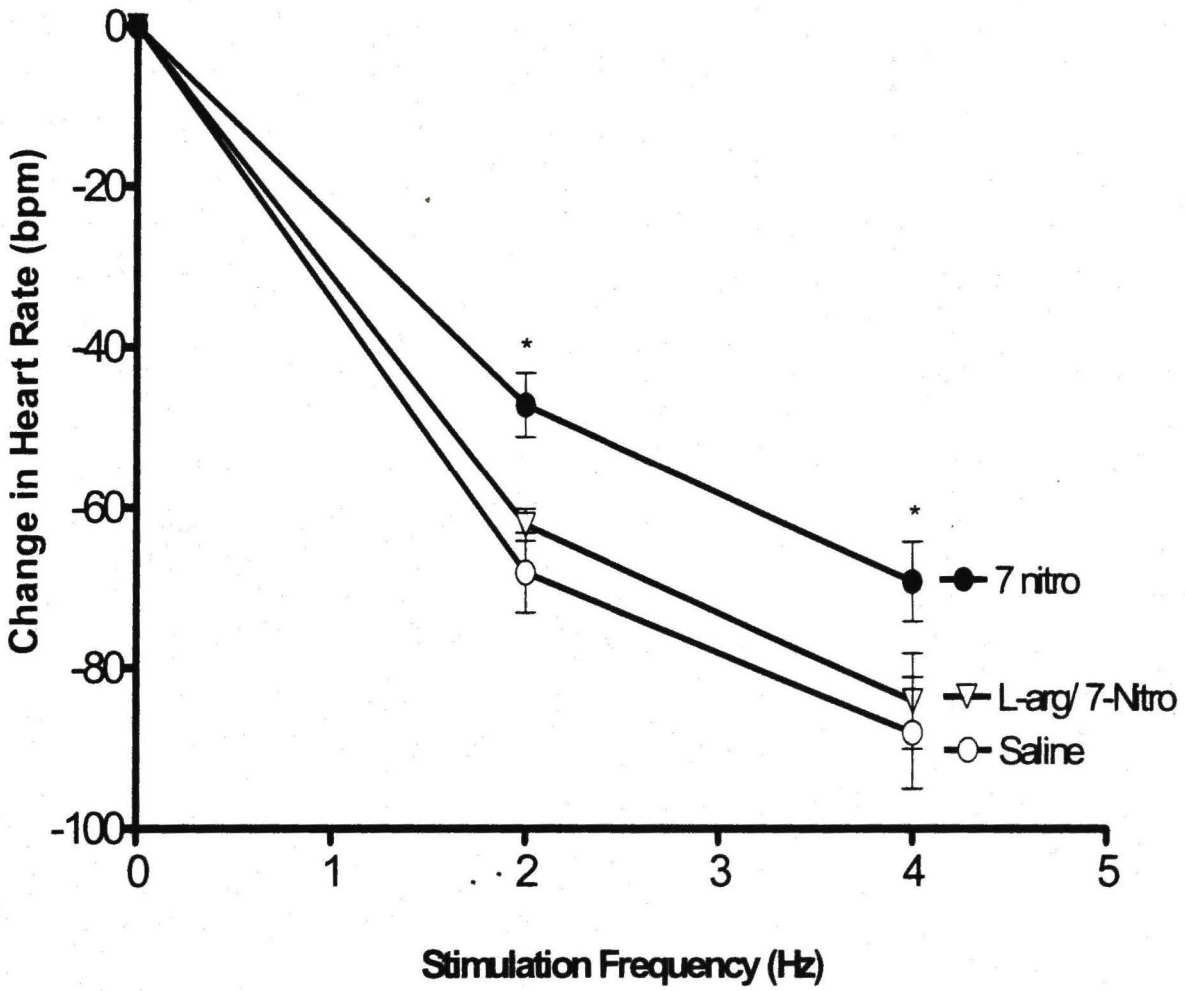
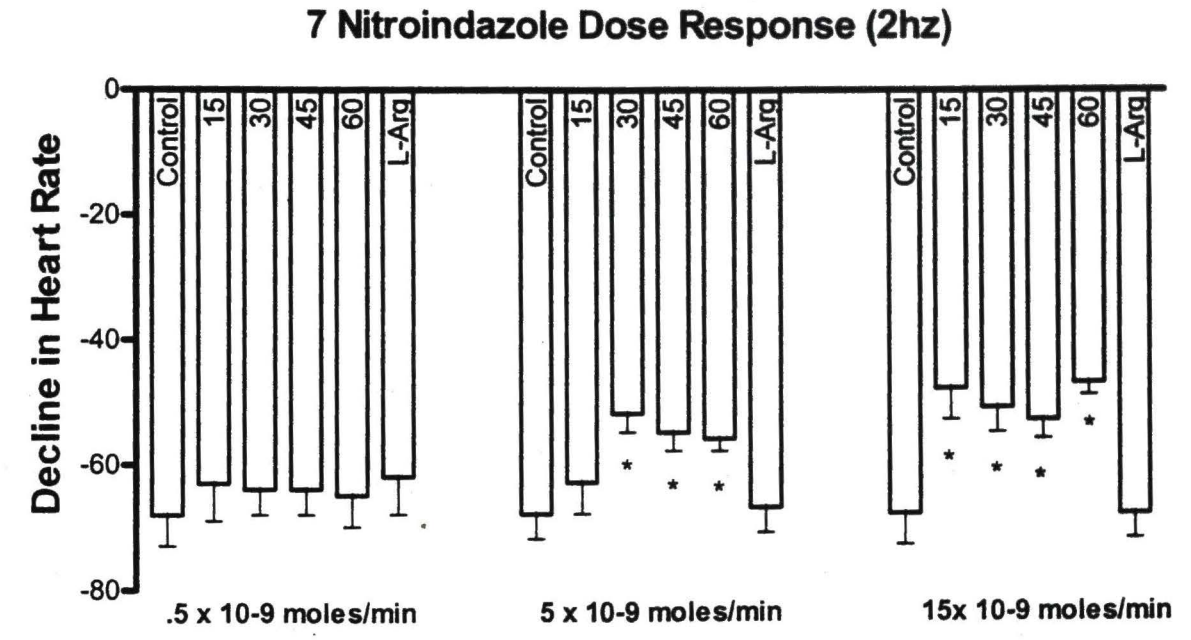

7 Nitroindazole attenuates vagal bradycardia
Change in Heart Rate (bpm)
0
-20
-40
-60
-80
-100
0
1
2
3
4
5
*
*
7 nitro
L-arg/ 7-Nitro
Saline
Stimulation Frequency (Hz)

Figure 3


7 Nitroindazole Dose Response (2hz)
Decline in Heart Rate
0
-20
-40
-60
-80
Control
15
30
45
60
L-Arg
.5 x 10-9 moles/min
5 x 10-9 moles/min
15x 10-9 moles/min

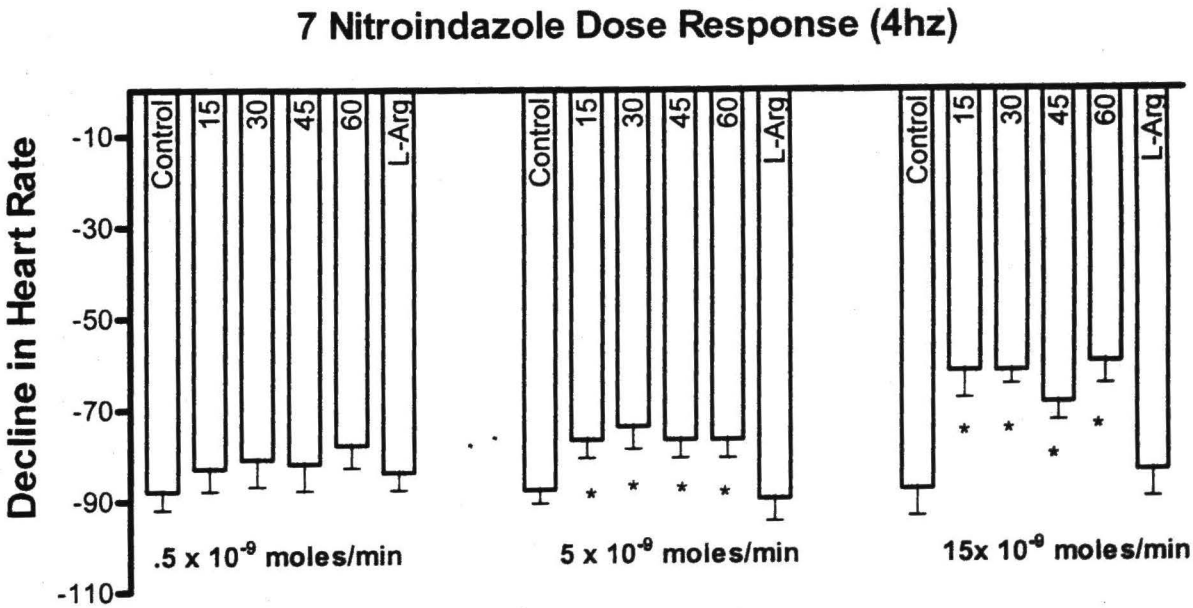

7 Nitroindazole Dose Response (4hz)
Decline in Heart Rate
-10
-30
-50
-70
-90
-110
Control
15
30
45
60
L-Arg
.5 x 10-9 moles/min
5 x 10-9 moles/min
15x 10-9 moles/min

Figure 4

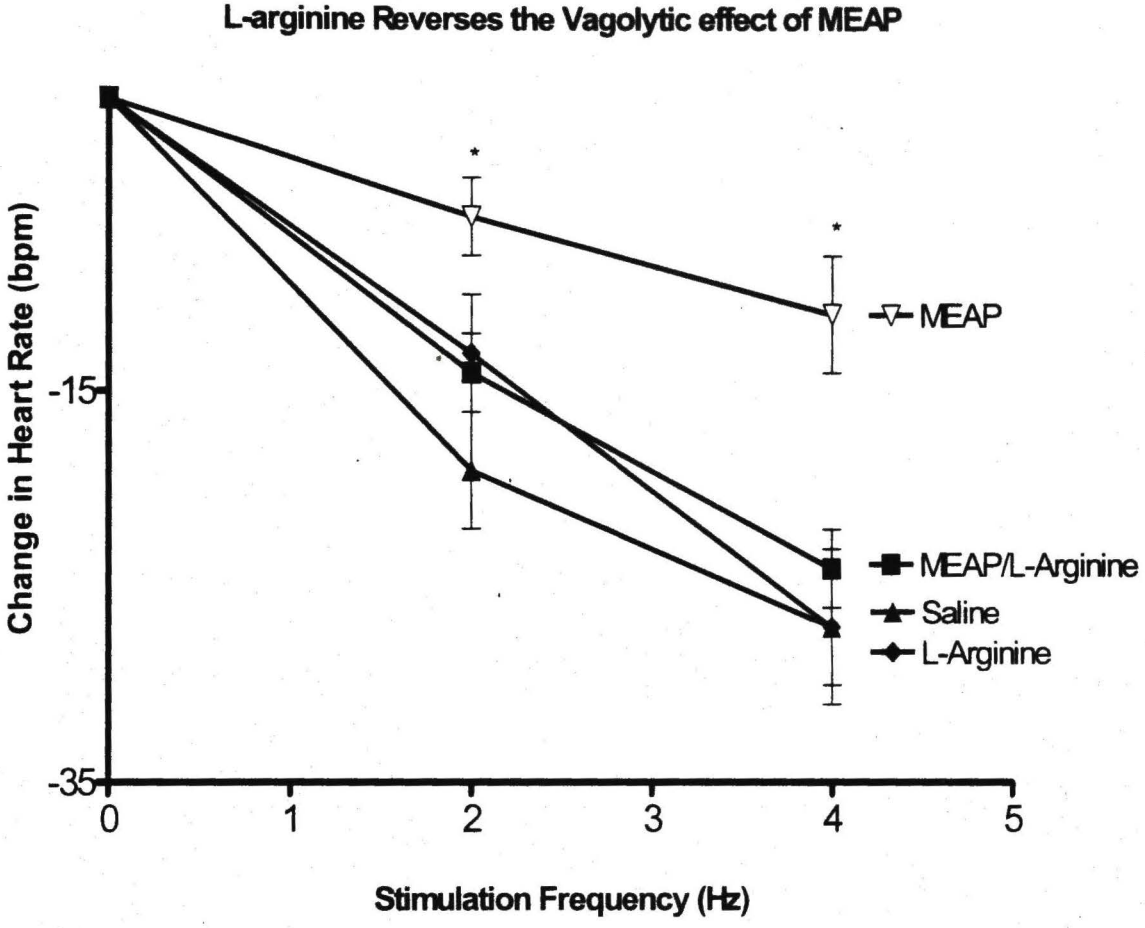
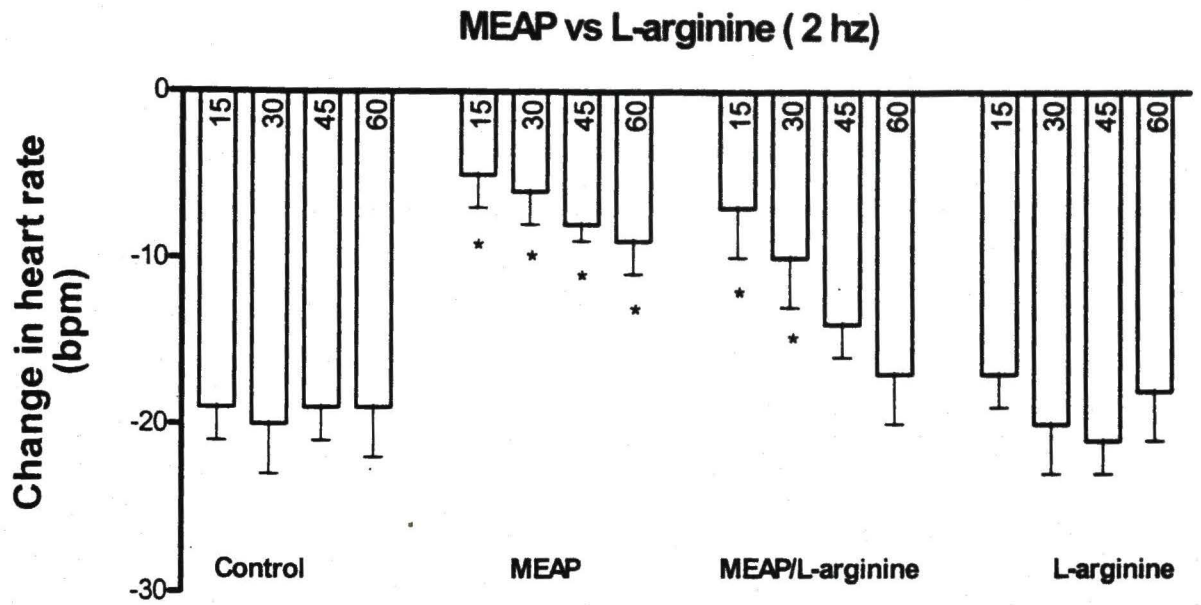

L-arginine Reverses the Vagolytic effect of MEAP
Change in Heart Rate (bpm)
-15
-35
0
1
2
3
4
5
*
*
MEAP
MEAP/L-Arginine
Saline
L-Arginine
Stimulation Frequency (Hz)

Figure 5


MEAP vs L-arginine (2 hz)
Change in heart rate (bpm)
0
-10
-20
-30
15
30
45
60
Control
MEAP
MEAP/L-arginine
L-arginine

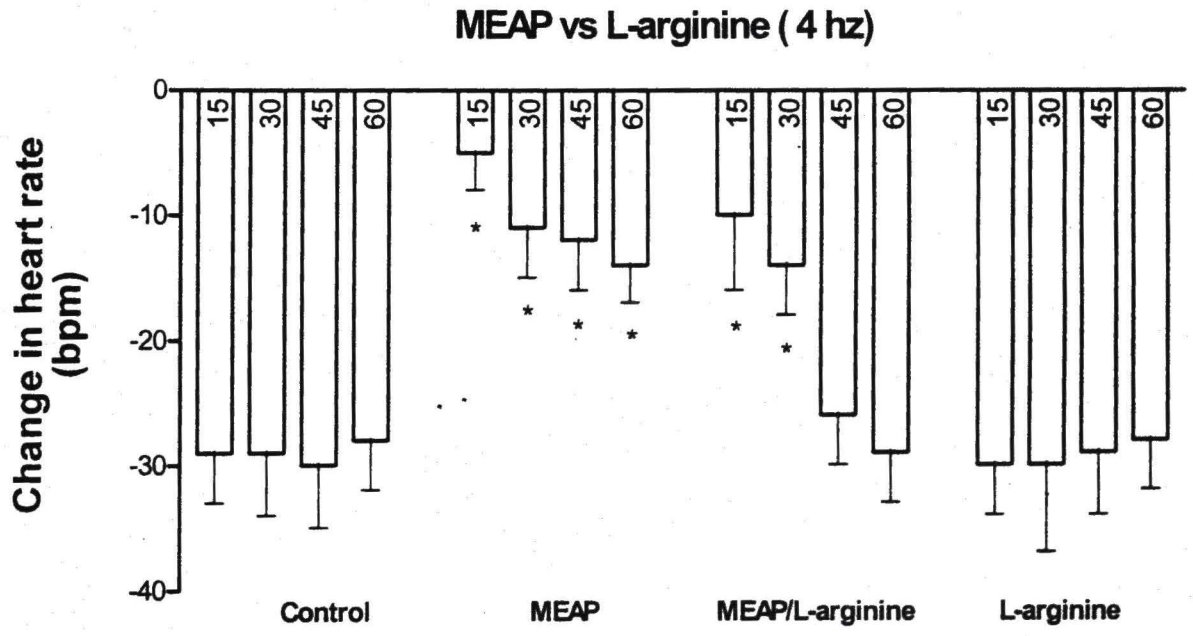

MEAP vs L-arginine (4 hz)
Change in heart rate (bpm)
0
-10
-20
-30
-40
15
30
45
60
Control
MEAP
MEAP/L-arginine
L-arginine

Figure 6

D-arginine does not reverse the vagolytic effect of MEAP

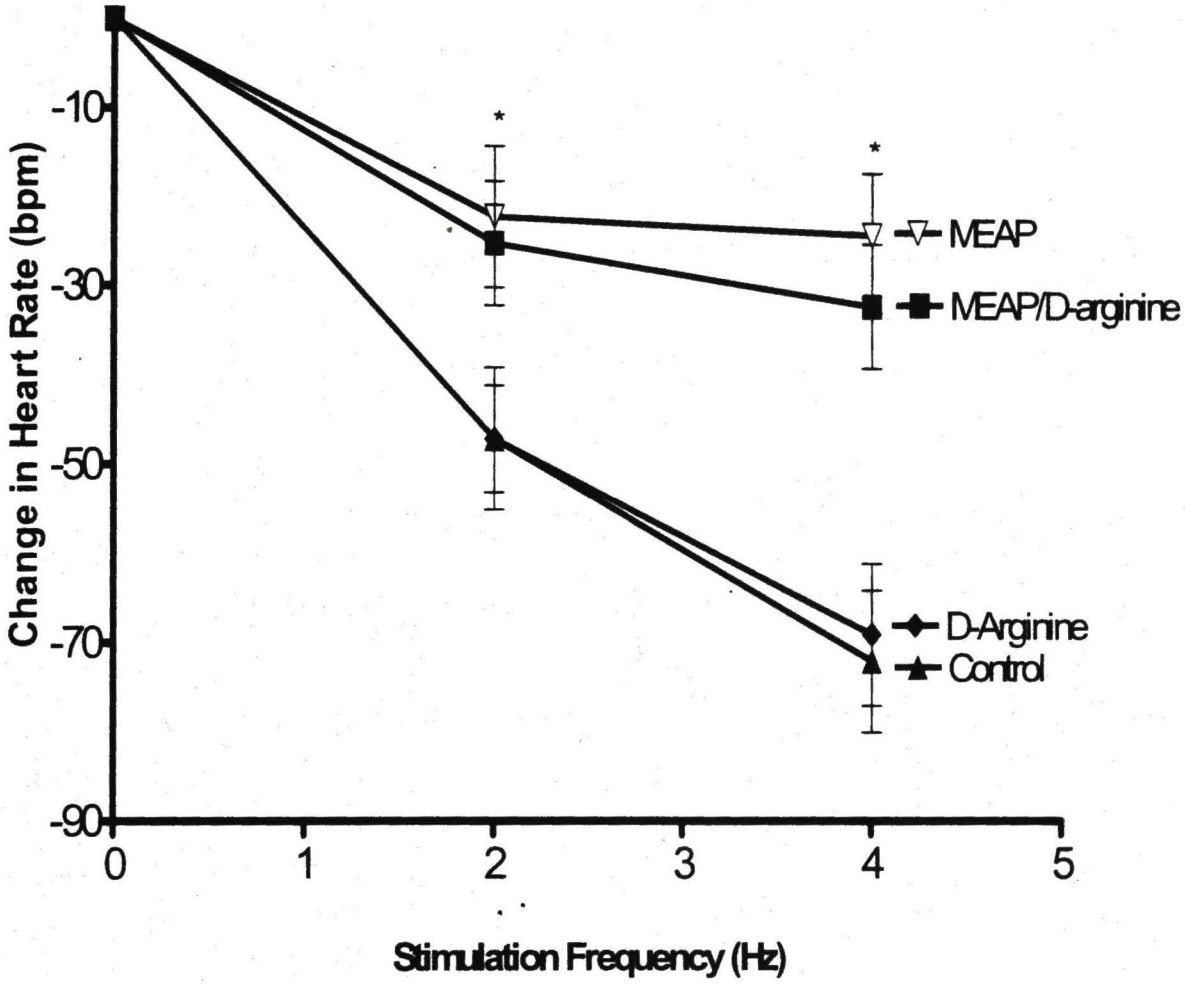
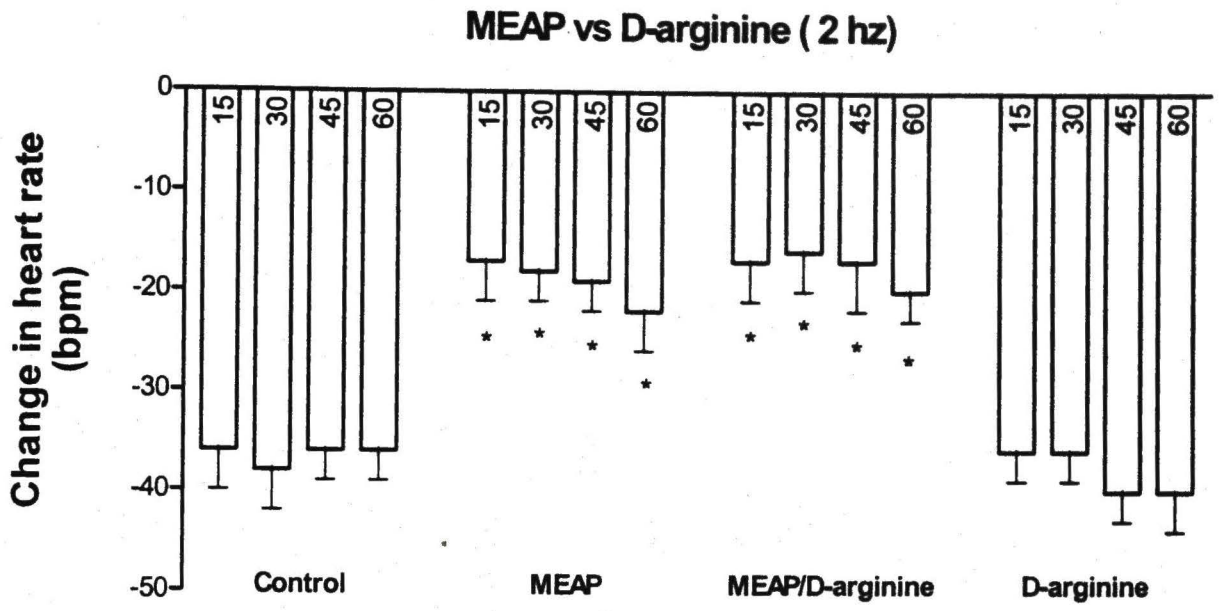


Figure 7


MEAP vs D-arginine (2 hz)
Change in heart rate (bpm)
0
-10
-20
-30
-40
-50
15
30
45
60
Control
MEAP
MEAP/D-arginine
D-arginine

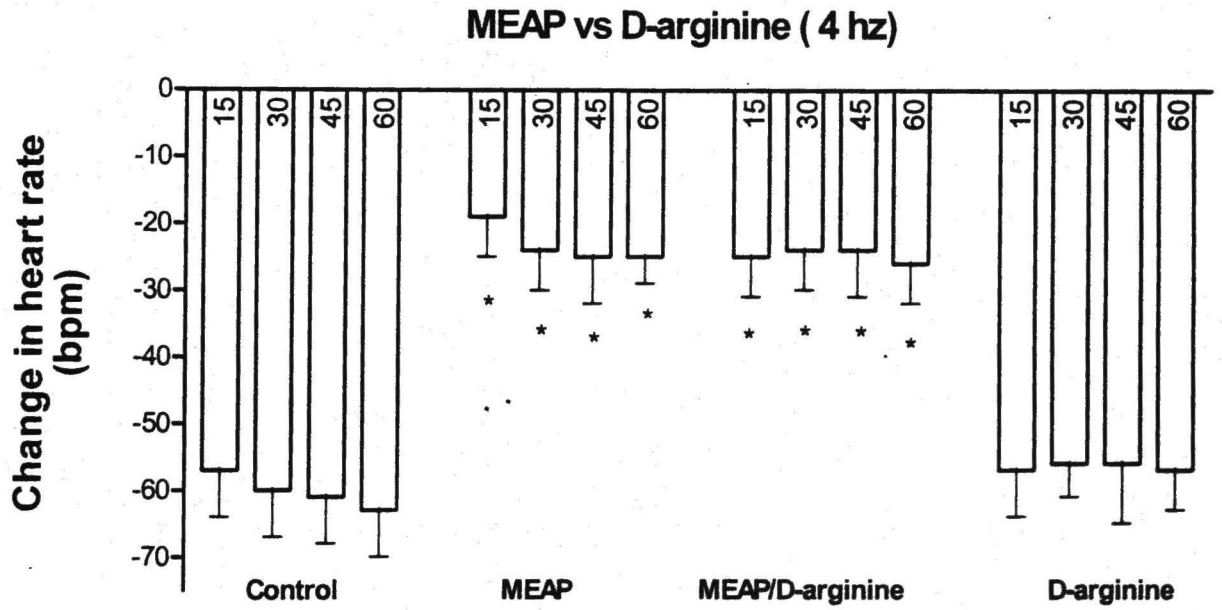

MEAP vs D-arginine (4 hz)
Change in heart rate (bpm)
0
-10
-20
-30
-40
-50
-60
-70
15
30
45
60
Control
MEAP
MEAP/D-arginine
D-arginine

Figure 8

SNAP Reverses the Vagolytic effect of MEAP

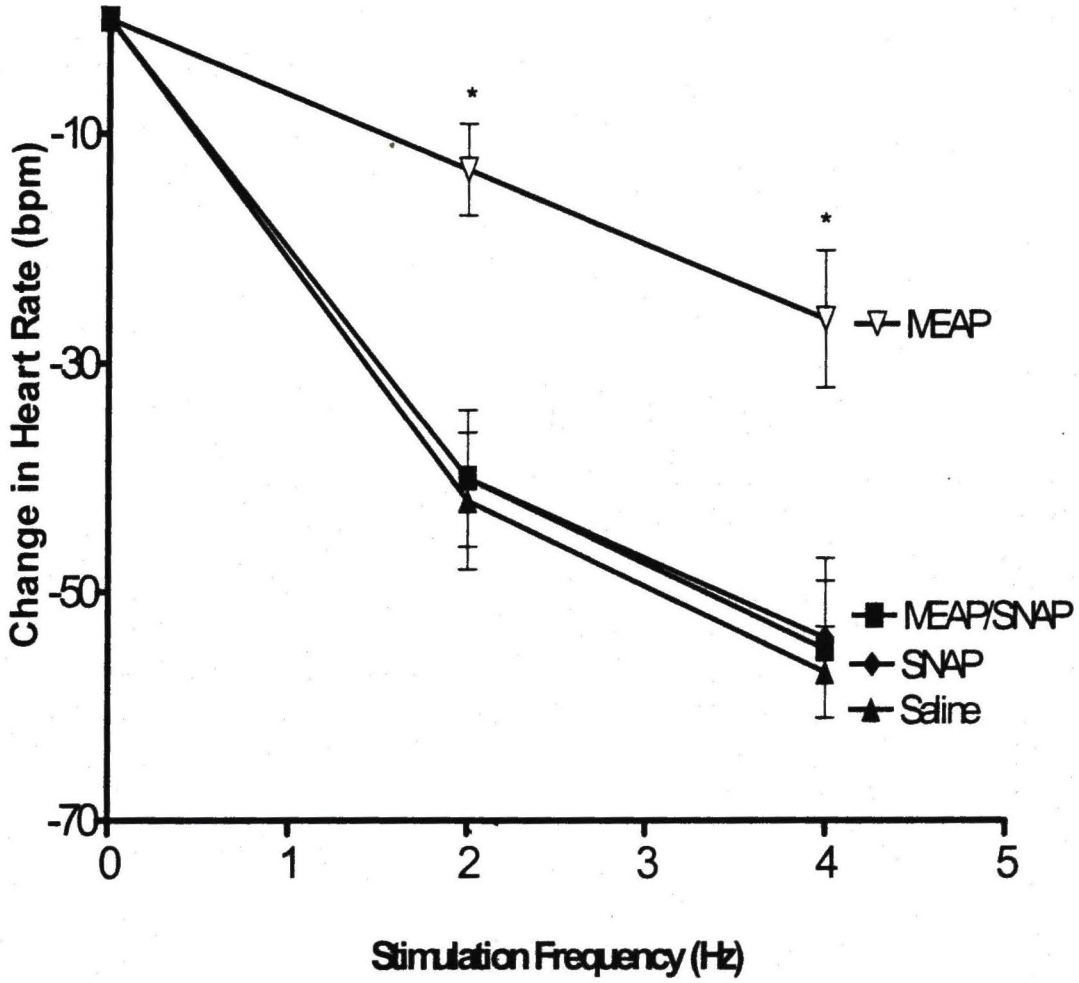
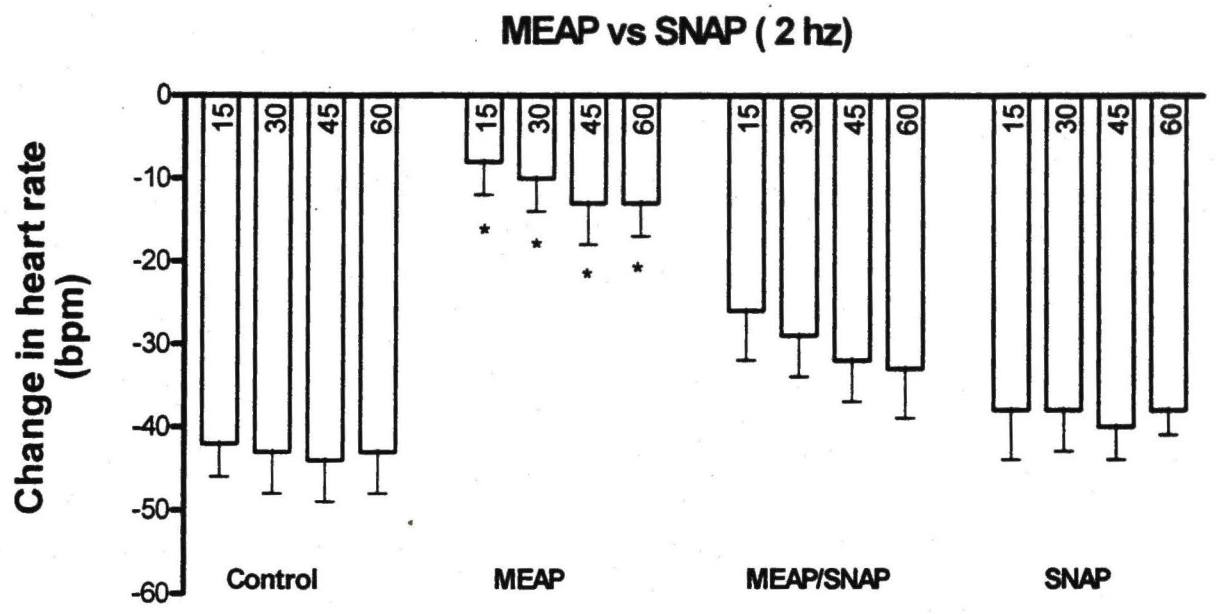


Figure 9


MEAP vs SNAP (2 hz)
Change in heart rate (bpm)
0
-10
-20
-30
-40
-50
-60
15
30
45
60
*
Control
MEAP
MEAP/SNAP
SNAP

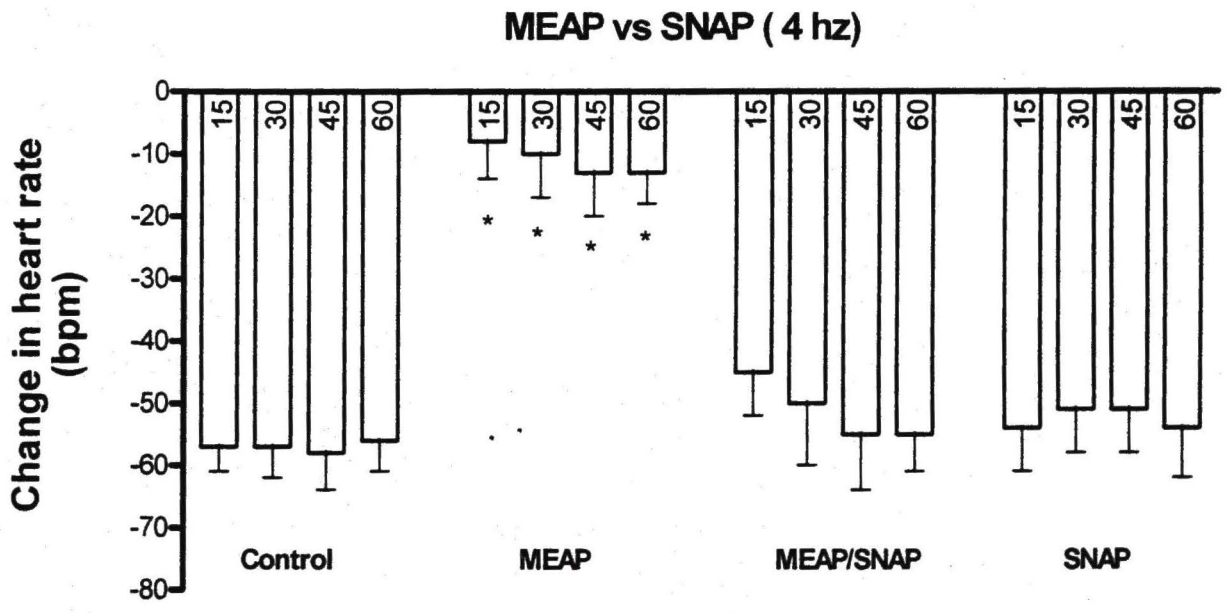

MEAP vs SNAP (4 hz)
Change in heart rate (bpm)
0
-10
-20
-30
-40
-50
-60
-70
-80
15
30
45
60
*
Control
MEAP
MEAP/SNAP
SNAP

Figure 10

cGMP Reverses the Vagolytic effect of MEAP

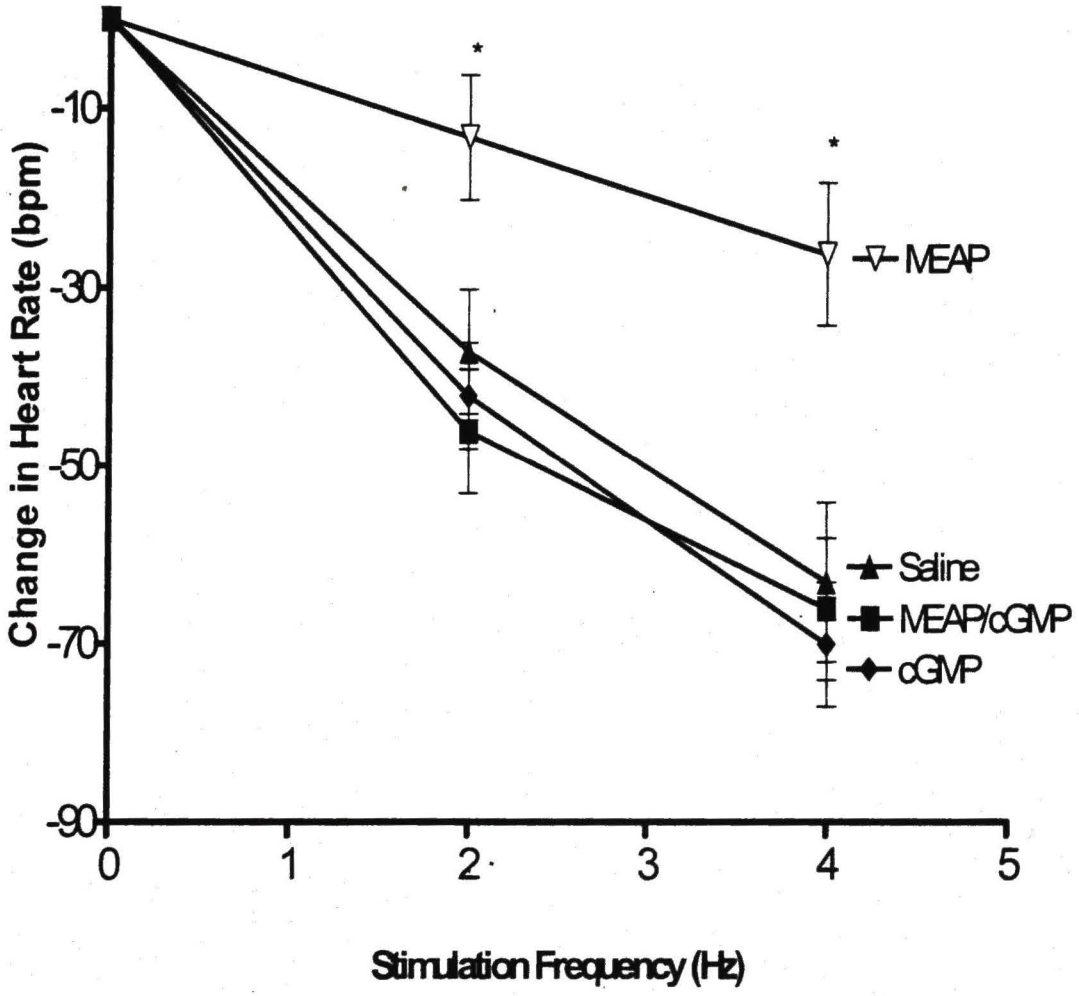
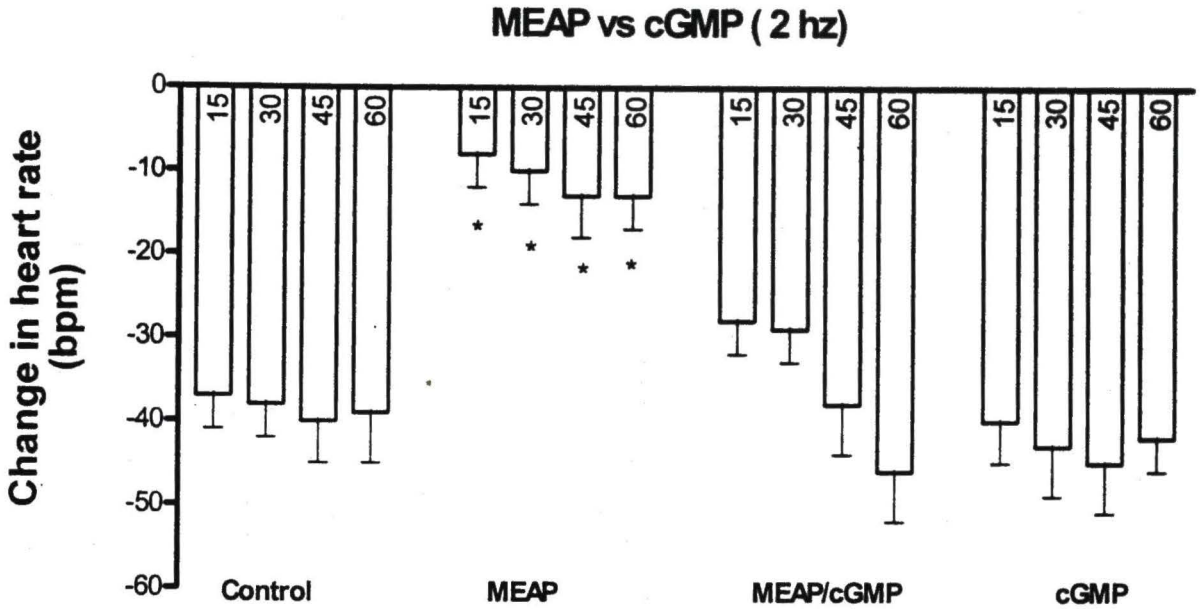


Figure 11


MEAP vs cGMP (2 hz)
Change in heart rate (bpm)
0
-10
-20
-30
-40
-50
-60
15
30
45
60
Control
MEAP
MEAP/cGMP
cGMP

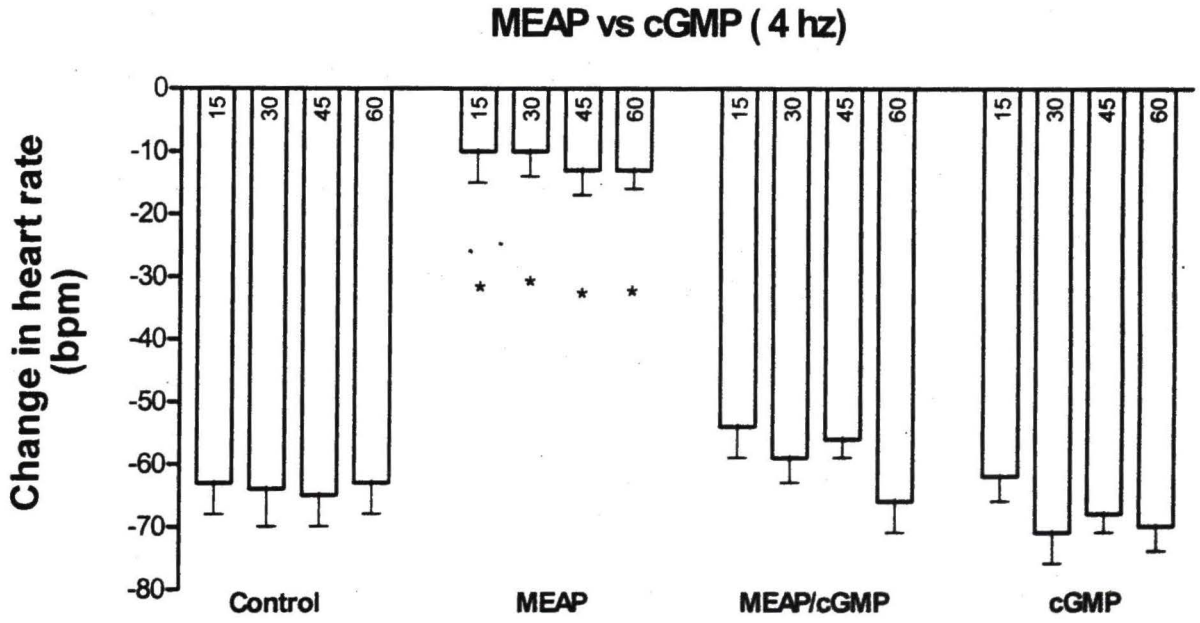

MEAP vs cGMP (4 hz)
Change in heart rate (bpm)
0
-10
-20
-30
-40
-50
-60
-70
-80
15
30
45
60
Control
MEAP
MEAP/cGMP
cGMP

Figure 12

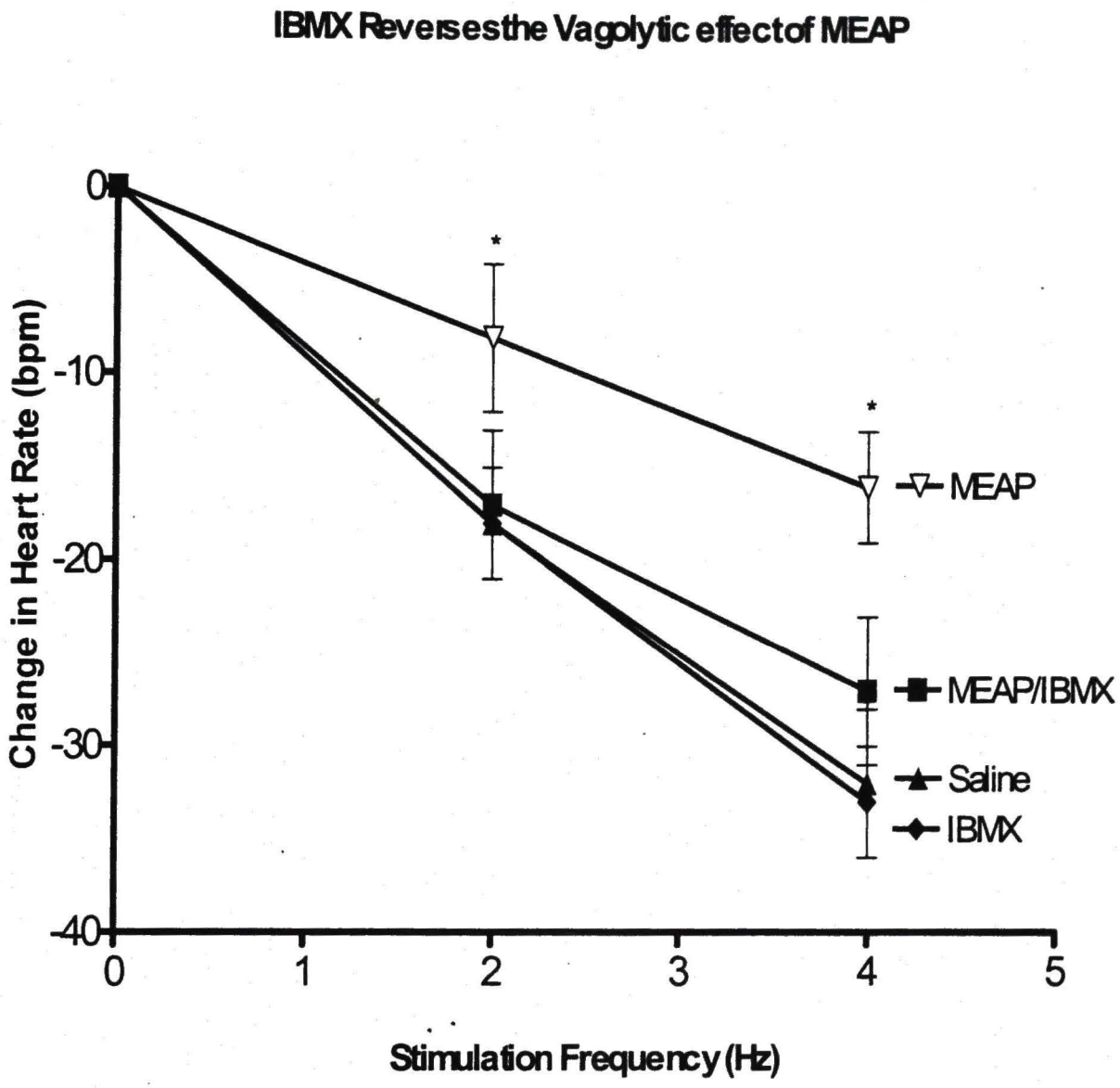

IBMX Reverses the Vagolytic effect of MEAP
Change in Heart Rate (bpm)
0
-10
-20
-30
-40
0
1
2
3
4
5
*
*
MEAP
MEAP/IBMX
Saline
IBMX
Stimulation Frequency (Hz)

Figure 13

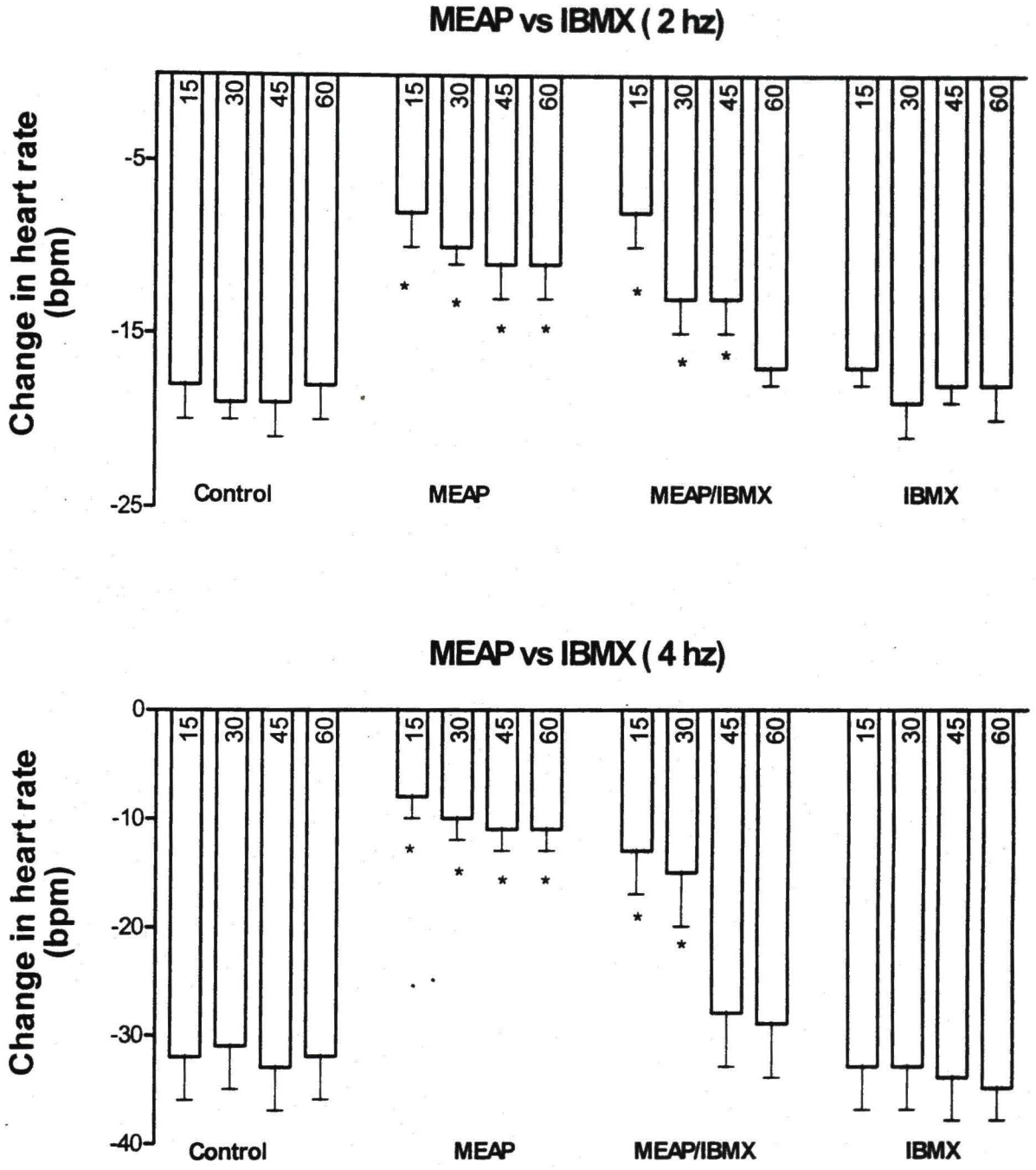
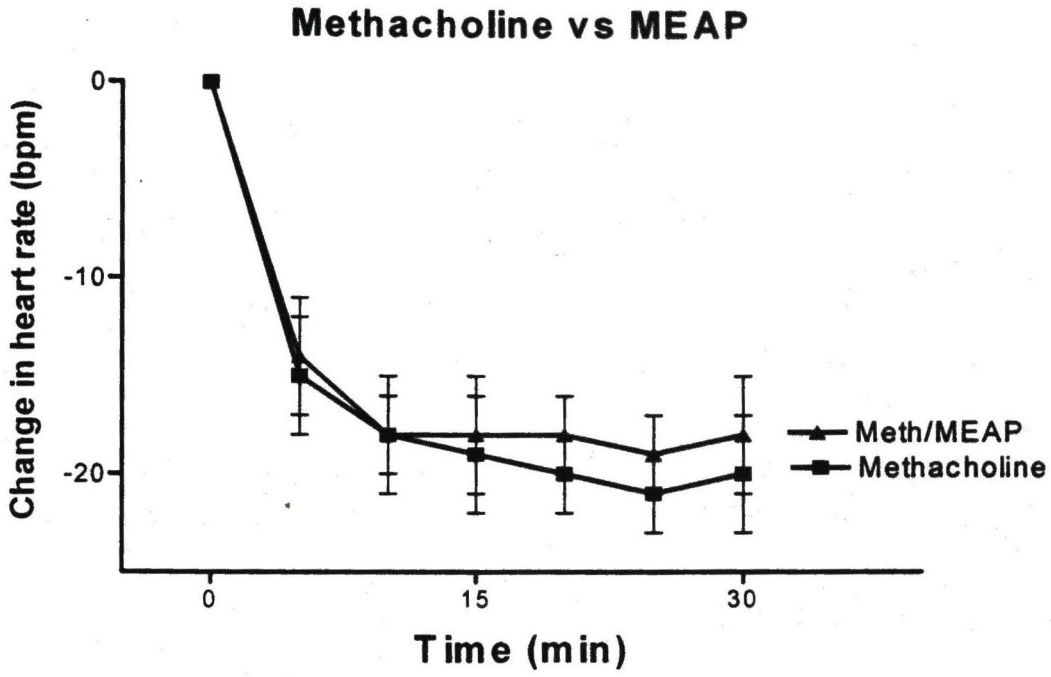

MEAP vs IBMX (2 hz)
Change in heart rate (bpm)
-5
-15
-25
15
30
45
60
Control
MEAP
MEAP/IBMX
IBMX
MEAP vs IBMX (4 hz)
Change in heart rate (bpm)
0
-10
-20
-30
-40
Control
MEAP
MEAP/IBMX
IBMX

Figure 14


Methacholine vs MEAP
Change in heart rate (bpm)
0
-10
-20
0
15
30
Time (min)
Meth/MEAP
Methacholine

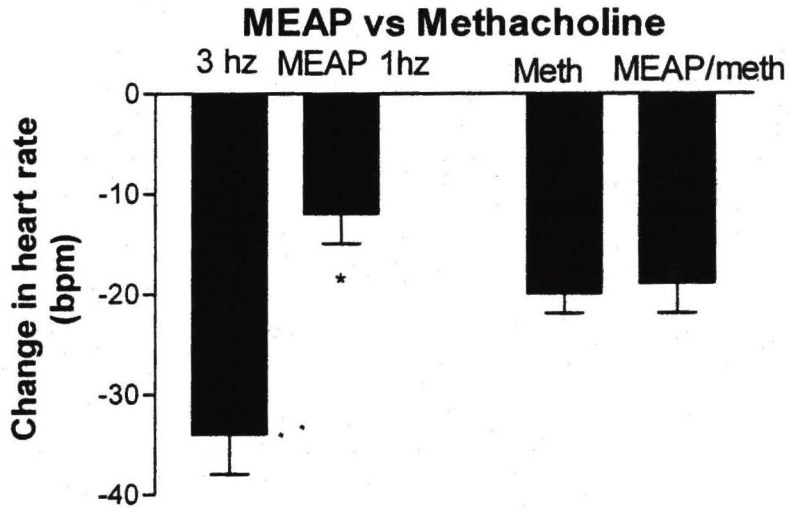
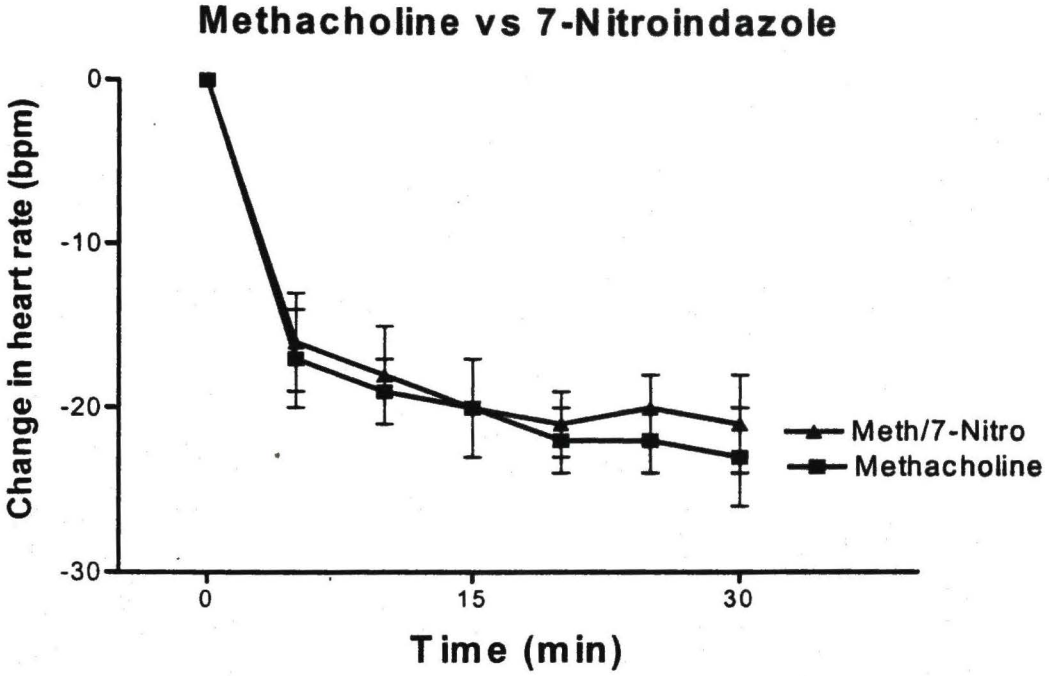

MEAP vs Methacholine
3 hz MEAP
1hz
Meth
MEAP/meth
Change in heart rate (bpm)
0
-10
-20
-30
-40
*

Figure 15


Methacholine vs 7-Nitroindazole
Change in heart rate (bpm)
0
-10
-20
-30
0
15
30
Time (min)
Meth/7-Nitro
Methacholine

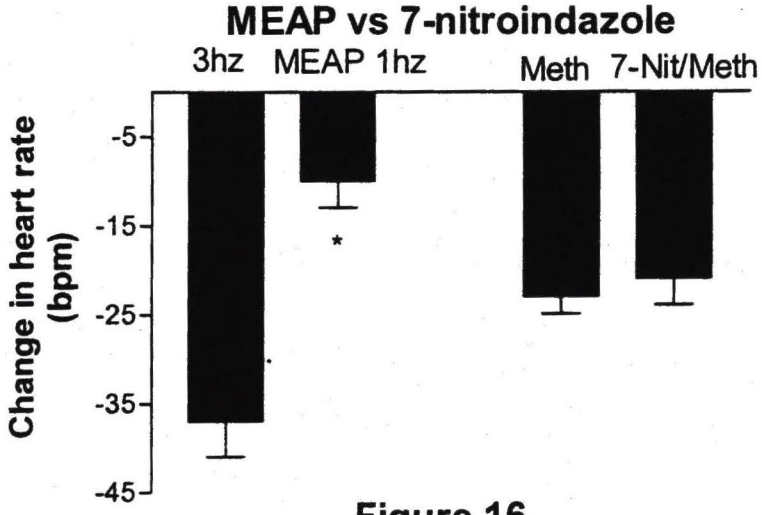

MEAP vs 7-nitroindazole
3hz
MEAP 1hz
Meth
7-Nit/Meth
Change in heart rate (bpm)
-5
-15
-25
-35
-45
*

Figure 16

CHAPTER IV

CONCLUSIONS

We began this endeavor with two basic questions: 1) which subtype of opiate receptor was mediating the vagolytic effect of MEAP and 2) was this vagolytic effect a result of the interruption of the NO-cGMP pathway within the sinoatrial node. The major findings of this work are:

1. The vagolytic effect of nodal MEAP was mediated by δ-2 opiate receptors in the sinoatrial node.

2. δ-1-opiate receptors in the sinoatrial node may be vagotonic.

3. The neuronal isoform of NOS in the canine sinoatrial node is an integral component of basal vagal transmission.

4. MEAP interrupts vagal bradycardia by interacting with the NOS-cGMP pathway in the sinoatrial node. The possible site of convergence is cAMP.

5. The interaction between MEAP and the NOS-cGMP pathway was not mediated by nodal muscarinic receptors and was likely prejunctionally located in vagal nerve terminals in the sinoatrial node.

The vagolytic effect of MEAP mediated by δ-2 opiate receptors may moderate the release of acetylcholine from parasympathetic nerve terminals. This scenario may protect the heart during intense vagal stimulation (vasovagal syncope) or smooth the transition between predominantly sympathetic and parasympathetic states. Nodal opioids may also be pathologic in selected circumstances since endogenous enkephalins have been reported to be elevated during hypertension and congestive heart failure. Inappropriate vagal transmission might increase sympathetic influences in the heart and possibly lead to rhythmic disturbances.

The activation of δ-1 opiate receptors may enhance vagal bradycardia. An increase in vagal transmission may be beneficial in acute myocardial ischemia. The local release of opioids may activate δ-1 opioid receptors and improve acetylcholine release in the ischemic area. The subsequent activation of muscarinic receptors would reduce myocardial contractile activity, oxygen consumption and the probability of tissue injury.

MEAP appears to exert its vagolytic action by interacting with the cAMP portion of the NOS-cGMP system in vagal nerve terminals in the sinoatrial node. This may be pathologic during heart failure and hypertension. Since the NO system is dysfunctional during these pathologies, a chronic rise in nodal MEAP could exacerbate this dysfunction by further reducing cAMP formation and decreasing vagal tone.

CHAPTER V

FUTURE STUDIES

The following studies are proposed to further clarify the cardiovascular effects of the MEAP and the Nitric Oxide System in the sinoatrial node:

1. Measure cAMP during treatment with NO or MEAP.

2. Use immunohistochemistry to determine the location and verify the types of opioid receptors harbored in the sinoatrial node.

3. Use immunohistochemistry to verify that opioid receptors and nitric oxide systems are colocalized in parasympathetic nerve terminals in the sinoatrial node.

4. Measure NO production during vagal nerve stimulation during normal conditions and opioid treatment.

5. Measure the release of acetylcholine during vagal stimulation with control and opioid treatments. This will directly determine the effect of opioid activation on the release of neurotransmitter.

6. Measure thmyocardial enkephalin during pathologic conditions such as congestive heart failure, hypertension, and myocardial infarction. This will determine if myocardial enkephalins are elevated during these conditions.

7. Use various kappa and mu agonists and antagonists to rule out their participation in the vagotonic effect produced by δ-1 agonists.

APPENDIX

The salient findings in this dissertation are: 1. δ-2 opioid receptors in the sinoatrial node are vagolytic, 2. δ-1 opioid receptors in the sinoatrial node are likely vagotonic, 3. Neuronal NOS in the sinoatrial node is a requisite part of vagal transmission, and 4. MEAP interacts with the cAMP component of the NOS-cGMP system in the sinoatrial node to modulate vagal bradycardia. As a point of clarity, several figures are provided to illustrate the possible mechanism for each finding.

First, the δ-2-opioid receptor attenuates vagal bradycardia by reducing the release of acetylcholine from vagal nerve terminals in the sinoatrial node (Figure 1). By coupling with Gi proteins, δ-2 receptor activation decreases the activity of adenylyl cyclase (AC) and leads to lower concentrations of cAMP in vagal nerve terminals. The decline in cyclic adenosine monophosphate (cAMP) reduces Ca^{2+} influx into the nerve terminals and decreases the vesicular release of acetylcholine. A decreased release in acetylcholine translates into the activation of fewer muscarinic receptors on nodal pacemaker cells and a less intense vagal bradycardia.

Second, the vagotonic effect of δ-1 receptors is mediated by increasing the release of acetylcholine (Figure 2). δ-1 receptors may accomplish this by coupling to Gs proteins and upon activation, increase the activity of adenylyl cyclase and elevating cAMP in

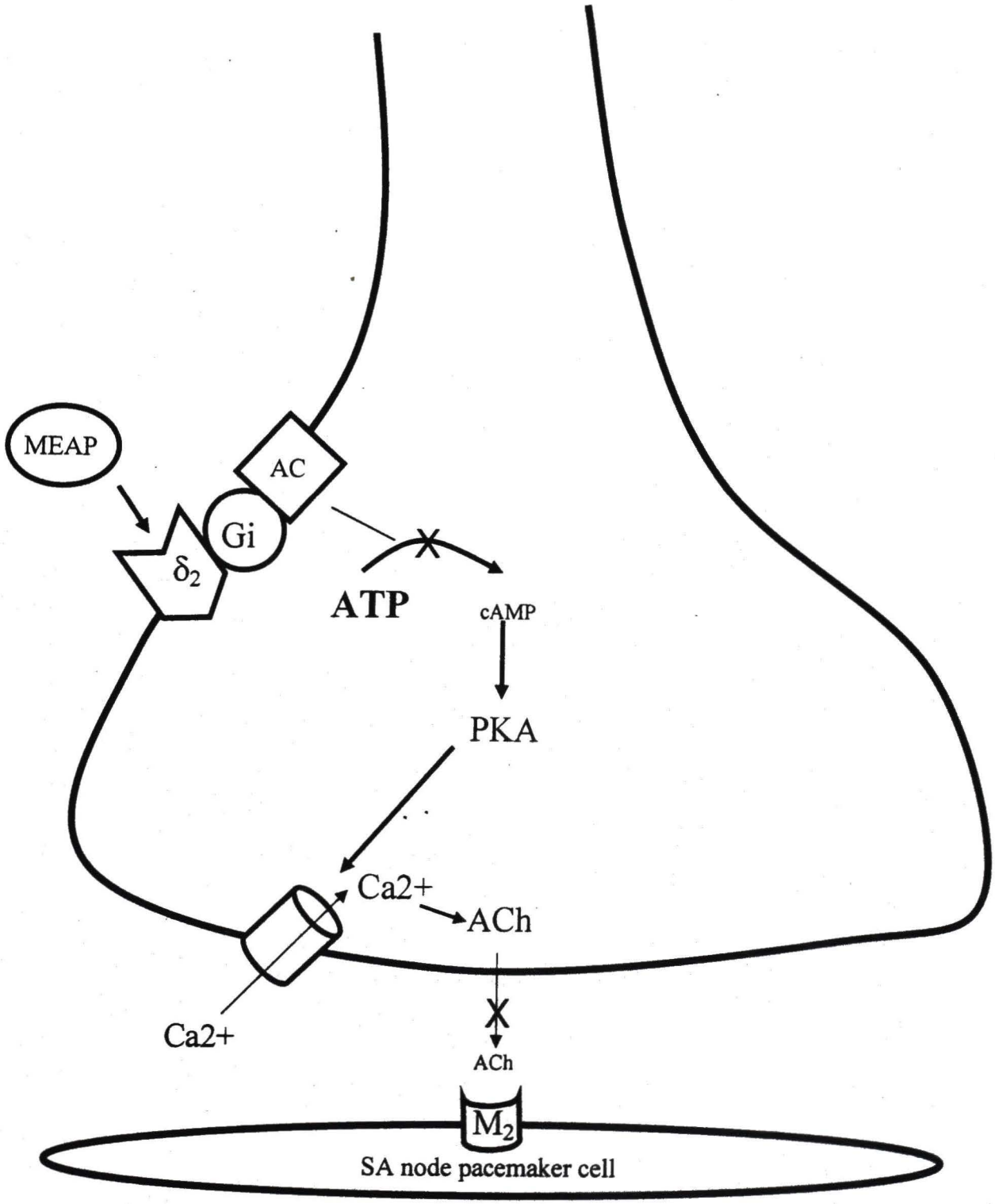
vagal nerve terminals. Overall, this would lead to an enhanced vagal bradycardia due to the increased release of acetylcholine and subsequent activation of muscarinic receptors on nodal pacemaker cells.

Third, NOS (nitric oxide synthase) is a requisite portion of vagal bradycardia since it aids in the release of acetylcholine from vagal nerve terminals by decreasing cAMP hydrolysis (Figure 3). When NOS is activated NO (nitric oxide) is formed. NO then acts on guanylyl cyclase (GC) to increase its ability to create cyclic guanosine monophospshate (cGMP). The cGMP molecule then activates protein kinase G (PKG) which inactivates phosphodiesterase 3 (PDE3). The normal activity of PDE3 is to hydrolyze cAMP. This inhibition allows the accumulation of cAMP and the subsequent influx of Ca^{2+} ions in vagal nerve terminals producing the vesicular release of acetylcholine and subsequent bradycardia.

Fourth, MEAP may interrupt this system by lowering cAMP concentrations normally maintained by the NOS-cGMP pathway by lowering adenylyl cyclase activity as described earlier in the appendix. Both opioids and nitric oxide may control vagal transmission by regulating the steady state concentrations of vagal cAMP and thus the release of acetylcholine (Figure 4). If as proposed, these two opposing mechanisms operate in different temporal domains. The relative activity of a constitutive NOS pathway may determine the responsiveness of vagal nerve terminals to opioids and any other effectors that modify the cyclase directly.

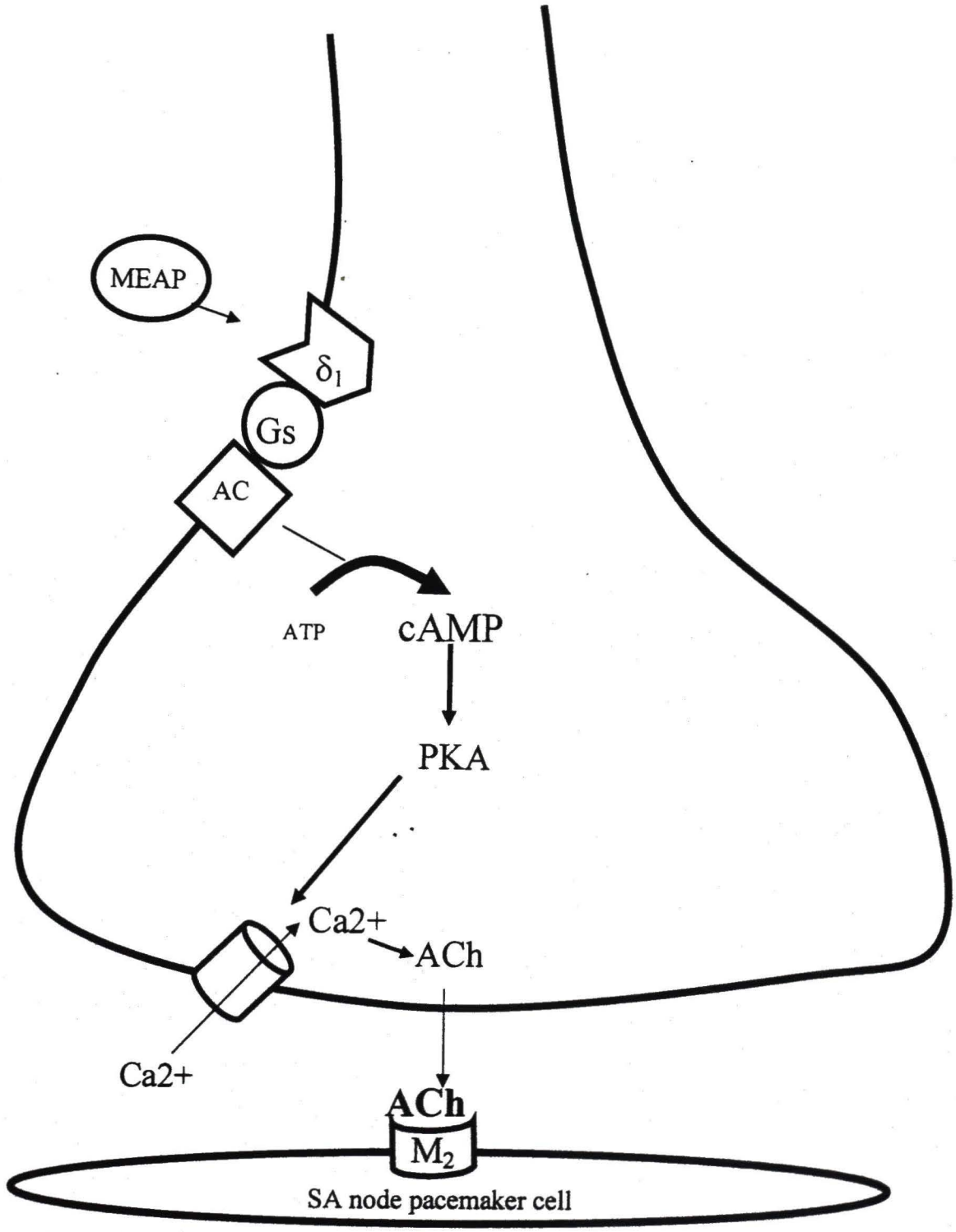
δ-2-opioid receptors are vagolytic

APPENDIX Figure 1



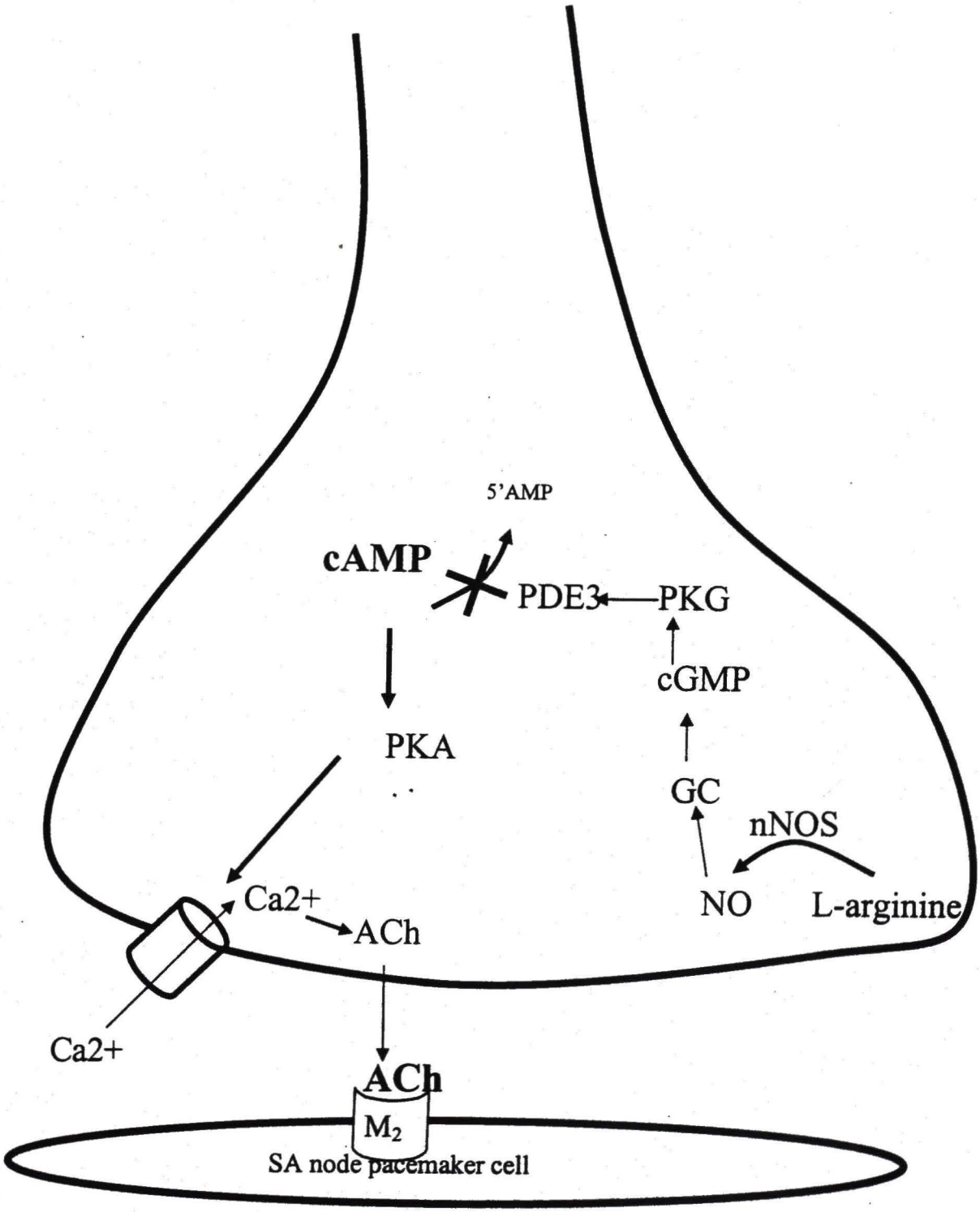
δ-1-opioid receptors are vagotonic

APPENDIX Figure 2



Neuronal NOS facilitates vagal bradycardia

APPENDIX Figure 3



MEAP and nNOS-cGMP pathway interaction
APPENDIX Figure 4