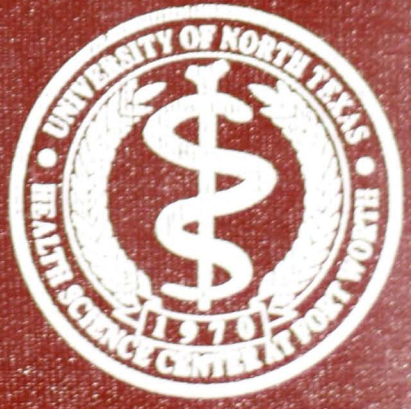
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Jackson, Keith E., Local Enkephalins Modulate Vagal Control of Heart Rate. Doctor of Philosophy (Biomedical Sciences), May 2001; 112pp; 7 tables; 22 figures; bibliography, 99 titles.

Endogenous opioids, such as enkephalins, were first investigated for their ability to modulate pain. A body of evidence now supports opioid actions in many facets of regulation, including the cardiovascular system. Our laboratory is particularly interested in the ability of opioids to modulate autonomic function. Specifically, the role of the endogenous enkephalin, methionine-enkephalin-arginine-phenylalanine (MEAP) was investigated to determine its ability to modulate parasympathetic function in the canine. To investigate MEAP's response in the sinoatrial (SA) node a novel application of microdialysis was employed, whereby microdialysis probes were fabricated as described by Dr. David Van Wylen (38), and implanted in the SA node.

After implantation of the probe, there was a significant attenuation of vagal function during the nodal application of MEAP. Specifically, vagally mediated bradycardia was reduced as compared to control, during the nodal application of MEAP. This inhibition of the vagus by MEAP was blocked by naltrindole, a selective delta antagonist. These data suggested that the vagolytic effects of MEAP were elicited via a delta opioid receptor.

To test the hypothesis that MEAP's effects were elicited through a delta opioid receptor mechanism, selective agonists and antagonists for the opioid receptors were utilized. An attenuation of vagal bradycardia was only observed during the infusion of a very selective delta opioid receptor agonist, deltorphin. A mu and kappa agonist showed no significant differences from control. Deltorphin was observed to elicit vagolytic effects in a similar concentration range as MEAP. However, deltorphin was more efficacious than MEAP. There was a significant attenuation of the deltorphin and MEAP's vagolytic effects, during the co-infusion of the selective delta antagonist, naltrindole. The mu and kappa antagonists were both ineffective. These data further demonstrate that the observed vagolytic effect is linked to a delta opioid receptor.

Endogenous MEAP

A series of experiments were undertaken to determine if endogenous MEAP could be demonstrated in the SA node and if so, was it similarly vagolytic. A preconditioning-like protocol was performed to produce intermittant local nodal ischemia to increase the local concentration of endogenous MEAP. The resulting MEAP was measured and was observed to be elevated during the periods of local nodal ischemia and return to control during reperfusion. Contrary to expectations an augmentation of vagal function was observed, during vagal stimulation. The augmented vagal bradycardia was only observed during ischemia, when MEAP was elevated and returned to control during each subsequent reperfusion. Therefore, there was a correlation between elevated MEAP concentrations and augmented vagal bradycardia. The delta antagonist, naltrindole, prevented the augmented vagal response, during nodal ischemia.

Glibenclamide, a selective K_{ATP} channel blocker, partially reversed the augmented vagal response. These data confirm that delta opiate receptors are involved in the augmented vagal bradycardia and that the mechanism may involve the activation of a K_{ATP} channel.

LOCAL ENKEPHALINS MODULATE VAGAL

CONTROL OF HEART RATE

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Original Articles

Napier, Leslie D., Amber Stanfill, Darice A. Yoshishige, **Keith E. Jackson**, Barbara A. Barron, and James L. Caffrey. Autonomic Control of Heart Rate in Dogs Treated Chronically with Morphine. *Am. J. Physiol.* 275 (*Heart Circ. Physiol.* 44): H2199-H2210, 1998.

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Keith E. Jackson, Martin Farias, Amber S. Stanfill, and James L. Caffery. Transient Ischemia Raises Enkephalin in the Sinoatrial Node and Improves Vagal Bradycardia. *Auton. Neurosci.* Submitted, 2000.

Abstracts and Invited Presentations

Effects of Oxidation on the Met-enkephalin-arg-phe (MEAP) Radioimmunoassay. October 18, 1997. Southwest Section of Society for Experimental Biology and Medicine, University of Texas at San Antonio.

Delta Opiate Receptor Antagonist Blocks the Vagolytic Effect of MEAP in the SA node. April 19, 1999. Experimental Biology 99, Washington, D.C.

Local MEAP Alters Sinoatrial Node Responses During Ischemia. July 11, 1999. INRC, Saratoga, New York.

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Pharmacologic Profiles Indicate Vagolytic Opiate Receptors in the S.A. Node are Delta Specific. International Narcotics Research Conference, 67, July 17, 2000.

Super DALDA Enhances Adrenergic Responses in the Dog. International Narcotics Research Conference, 66, July 17, 2000.

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CHAPTER I

INTRODUCTION

The investigations described in this dissertation were performed to examine the local sinoatrial nodal physiology of the canine heart. In this light, a novel application of the microdialysis technique was utilized to explore the parasympathetic nerve interactions in the nodal environment. Recent investigations in our laboratory indicated that there was a significant attenuation of vagal control of heart rate (9) during brief infusion of the node with methionine-enkephalin-arginine-phenylalanine (MEAP). The investigators hypothesized that a delta opioid receptor was involved, but the exact mediator of the response and mechanism of action of this opioid effect remained unclear (9, 10). Previous studies suggested that opioids were involved in the beneficial effects of ischemic preconditioning (12, 30, 37, 50). The cardioprotective effects of opioids were linked to a delta-1 receptor (48, 49) and a potassium ATP (K_{ATP}) channel mechanism (18, 20, 48).

The objective of this dissertation was two fold: first, to delineate which opioid receptor is responsible for MEAP's vagolytic effect and second, to examine if the effects of exogenously administered MEAP correlated with the effect of

endogenously produced MEAP. The studies which follow examined MEAP's vagolytic role through exogenous infusion of both agonists and antagonists selective for the three opioid receptors. To correlate the exogenous and endogenous effects of MEAP, a "preconditioning-like" protocol was conducted in the canine heart to increase MEAP concentrations in that local nodal environment.

REVIEW OF RELATED LITERATURE

Endogenous Opioids

Opium as extracted from the opium poppy, *Papaver Sominiferum*, is one of the oldest medications known. Opium's medicinal properties as an analgesic and anti-diarrheal were already recognized by the ancient Sumerians (4000 BC) and Egyptians (2000 BC) (6). The main active ingredient in opium is the alkaloid, morphine, which remains the most effective and widely used analgesic worldwide. Unfortunately both opium and morphine are addictive and both have similar abuse potential. As a point of clarity, the word opiates originally referred to alkaloids derived from the juice of opium poppy. Opioids (literally "opium-like") were intended to refer to compounds other than opium alkaloids having pharmacological effects similar to those of opium. Today the terms opiates and opioids are often used interchangeable given their common ability to modulate pain through similar receptor activation.

The search for the mechanism of action of morphine combined with the search for new painkillers without abuse potential produced many disparate results. In 1942,

Weiijlard et al synthesized nalorphine (N-allylnormprphine), which had limited analgesic activity and subsequently became (6) the first opiate antagonist. This compound could reverse the respiratory depression produced by morphine and precipitate the abstinence syndrome in addicts (6). Nalorphine was eventually found to have mixed agonist and antagonist effects. The variety of actions mediated by the opium alkaloids (analgesia, euphoria, tolerance and physical dependence) combined with inconsistencies in the actions of synthetic opioid agonists and antagonists was best explained by the existence of different receptor classes.

In 1973, Pert et. al, Simon et al and Terenius identified almost simultaneously that there were stereospecific opiate binding sites in the central nervous system (42, 46, 54). These opiate receptors were later found to be distributed nonuniformly in the central nervous system suggesting that these receptors might be targets for neurotransmitters, i.e. endogenous opiates (22, 42). Akil et al demonstrated that footshoock stress in rats induced analgesia, which was partially reversed by the opioid receptor antagonist, naloxone (6). They suggested that stress must induce the release of endogenous compounds pharmacologically similar to the analgesic, morphine.

Kosterlitz et al observed that brain extracts contained an agent that like morphine inhibited acetylcholine release in the guinea pig ileum (27). The inhibition of acetylcholine from nerves in the guinea pig ileum was reversed by naloxone

administration, providing additional evidence that endogenous opioids were involved. The factors responsible for this inhibition of acetylcholine release (26), were isolated and identified as two similar pentapeptides, namely methionine-enkephalin (Tyr-Gly-Gly-Phe-Met), and leucine-enkephalin (Try-Gly-Gly-Phe-Leu). The methionine-enkephalin sequence was present on the N-terminus of another peptide, beta-endorphin (6), a fragment of beta lipotropin that had been isolated several years earlier from pituitary extracts (5, 22). Like the enkephalins, beta-endorphin proved to have a high affinity for brain opioid receptors, providing evidence for its intrinsic opiate activity.

Processing of Opioid Peptides

Proenkephalin was first discovered in bovine adrenal cortex, where enkephalin biosynthesis was elucidated by Udenfriend et al (45). Proenkephalin was later cloned from bovine and human tissues. The enkephalins were soon discovered to be processed from this larger precursor protein, proenkephalin (39). Proenkephalin contains four sequences of methionine-enkephalin, one leucine-enkephalin, one methionine-enkephalin-Arg^6-Phe^7 and one methionine-enkephalin-Arg^6-Gly^7-Leu^8 (2, 21, 29). The enkephalins are located between pairs of basic amino acids. The basic amino acids represent sites for selective cleavage of proenkephalin into several biologically active constituents. Several different post-translational intermediates are possible following selective cleavage by different proteases. Such circulating

intermediate sized peptides could represent inactive zymogens that are subsequently processed to active enkephalin within target tissues (41).

Endogenous Opioid Receptors

Martin et. al. provided the first conclusive evidence for the existence of opioid receptors (35). Through neurophysiological and behavioral analysis, they examined cross-tolerance among different opioid compounds. Based on their findings they suggested the existence of different opioid receptor classes. The three opioid receptor classes were each named for their respective agonist, namely, mu (morphine), kappa (ketocyclazocine), and sigma (N-allylnormetazoncine) (35). The sigma receptor is no longer recognized as an opioid receptor (6).

After Hughes et. al., discovered the enkephalins (22), investigators were interested in determining for which of the opioid receptors the enkephalins were more selective (34). Kosterlitz et al demonstrated that the enkephalins were more active than morphine in inhibiting contraction of the mouse vas defferens, although morphine was more efficacious at inhibiting guinea pig ileum contraction (6). These disparate observations then led to the hypothesis that another opioid receptor was present in the mouse vas defferens (34), which they named the delta receptor. In the years following the investigations by Kosterlitz et. al., additional more selective ligands for the mu, kappa and delta receptors were developed (6). The results of the various anatomical, physiological, and pharmacological studies have demonstrated that there

are subclasses within each group, for example delta-1 and delta-2 subtypes in the delta class.

Opioids are involved in a wide spectrum of physiological processes. Most of these opioid actions are accomplished through opioid receptor mediated neuromodulation via the inhibition of neurotransmitter release (8, 13, 14, 15).

Opioids and the Parasympathetic Nerve

The moment to moment control of heart rate rests almost exclusively with the vagus nerve, which serves as an ever-present brake on the heart (4, 11). Instantaneous changes in heart rate are primarily achieved by increasing or withdrawing the vagal effect. The practical significance of the vagus is illustrated by the fact that patients who regain vagal control of heart rate soon after suffering heart attacks are far more likely to be alive three years later (25).

Our laboratory has long been interested in the interaction of cardiac enkephalins and their effects within the cardiovascular system. Opioids, which were first discovered for their ability to modulate pain, have now been shown to have a number of other functions (8, 10, 38). Some of these opioid functions include the regulation of neurotransmitter release and various associated cardiovascular modulatory functions, such as inhibition of vagally mediated increases in coronary blood flow, and vagally mediated bradycardia (8, 10).

MEAP is an endogenous opiate derived from the C-terminal sequence of the larger precursor molecule proenkephalin (52). MEAP is an important myocardial regulator of vagal control of the heart. MEAP is one of the most abundant opiate peptides in the myocardium (2) and when administered systemically it immediately and reversibly blocks vagal control of heart rate (9), contractile activity and coronary blood flow (8). We have been able to find that MEAP 1) is produced within the substance of the heart (21, 29, 52), 2) is capable of inhibiting vagally mediated bradycardia (9, 26), and 3) increases in the heart and circulation during circulatory stress, such as hypotension (36). Although plasma MEAP concentration increases dramatically during circulatory stress, MEAP's rapid degradation and dilution en route to the circulation complicate the evaluation of local peptide changes in the myocardium (41). In this light, the vagolytic effect of MEAP was then localized to either the intracardiac parasympathetic ganglion or the nearby sinoatrial node (9). Since the sinoatrial node artery supplies both the sinoatrial node and the intracardiac ganglion, separating out the site of action for MEAP has been extremely difficult. We have recently developed a novel approach using microdialysis for the examination of the exact site of the MEAP mediated vagolytic effect (1, 56). We were able to reproducibly place microdialysis probes within the substance of the sinoatrial node with no significant deterioration in nodal function. The functional location of the probe was verified when norepinephrine was infused into the probe and a significant tachycardia was observed (heart rate increased by 40 bpm). After placement of the

probes, direct stimulation of the vagus nerve produced a significant bradycardia and in related studies, stimulation of the sympathetic input to the node, produced a brisk tachycardia. Overall, these observations demonstrate that autonomic function was not impaired following insertion of the microdialysis probe.

Microdialysis helped show that MEAP's effect was directly localized within the sinoatrial node, with little to no action of MEAP on the intracardiac ganglion (16). This vagolytic effect was reversed by infusion of the nonselective opioid antagonist, diprenorphine (16), suggesting that MEAP's vagolytic effect was linked to an opioid receptor mechanism.

Opioids and Preconditioning Ischemia

A number of physiological circumstances have been associated with increasing the enkephalin levels in the body including hemorrhagic hypotension, inhibition of sympathetic outflow, and local ischemia (32, 36). We chose to raise endogenous MEAP by lowering blood flow transiently using a "preconditioning-like" protocol. This study employed a commonly used protocol of four ten minute cycles of reduced blood flow intermixed with ten minute cycles of reperfusion. This preconditioning-like period was followed by thirty minutes of low flow and thirty minutes of reperfusion. This protocol was identical to preconditioning protocols utilized in other studies. However, the current studies described in this manuscript did not examine preconditioning outcomes in the sinoatrial node, therefore this protocol was labeled

"preconditioning-like." This procedure was selected because it produced only local alterations in the sinoatrial node, whereas hemmorhagic hypotension and sympathetic withdrawal would have each precipitated more global compensations. Furthermore, opioids have been linked in previous studies to the cardiac protection rendered by preconditioning in the ventricular myocardium (17, 18, 30, 49).

Ischemic preconditioning is a phenomenon in which one or several cycle(s) of brief ischemia and reperfusion protects the myocardium against cell injury caused by subsequent prolonged ischemia (13). Given the similarity between the protocol utilized in our investigations and preconditioning ischemia, it was reasonable to briefly review the role of opioids in the preconditioning literature. Ischemic preconditioning was linked to a delta receptor mechanism in rats, since 1) morphine, a non-selective opioid agonist, reduced infarct size as a percent of area at risk (IF/AAR), similar to preconditioned rats, 2) naltrindole, a selective delta antagonist, blocked morphine preconditioning and 3) naltrindole blocked ischemic preconditioning (49). Schultz et al examined the opiate effect to determine if it was centrally or peripherally mediated (50). They compared the ability of the opiate antagonist naloxone and naloxone methiodide to eliminate the cardioprotection observed during ischemic preconditioning. Naloxone methiodide, which does not cross the blood-brain barrier, reduced the preconditioning effect similar to naloxone, which freely crosses into the central nervous system (50). Since both naloxone and

naloxone, methiodide were able to reverse the effects of preconditioning, the participation by opiates appeared to be mediated peripherally.

The preconditioning study above was repeated in intact rabbits and in isolated rabbit hearts by Chien et al to confirm that opioid preconditioning was a peripherally mediated effect (12). Both naloxone methiodide and naloxone hydrochloride blocked the cardioprotection rendered by ischemic preconditioning in intact rabbits (12). As further proof of the peripheral action of opioids, Chein et al (12) observed that naloxone hydrochloride also effectively blocked ischemic preconditioning in isolated rabbit heart. In subsequent studies, opioid preconditioning effects in rat hearts were found to be mediated by a delta-1 receptor (47). Naltriben methanesulfonate (NTB), a selective delta-2 antagonist, and 7-benzylidenenaltrexone (BNTX), a selective delta-1, antagonist were given prior to preconditioning in rats. BNTX was found to block the reduction in percent infarct size, while [D-Ala(2), N-MePhe(4), Gly-ol(5)] enkephalin (DAMGO, mu opioid receptor agonist), NTB, beta-funaltrexamine (beta-FNA, mu opioid receptor antagonist), and norbinaltorphimine (nor-BNI, kappa-opioid receptor antagonist) were ineffective (47). Therefore, it was concluded that opioid mediated ischemic preconditioning employed a delta-1 receptor mechanism.

ATP-sensitive potassium channels and preconditioning ischemia

The ATP-sensitive potassium channel (K_{ATP} channel) in cardiomyocytes has also been implicated in the cardioprotective effects elicited through myocardial preconditioning (18, 30, 48). Normally inhibited by intracellular ATP, the K_{ATP} channel can become activated by conditions, such as ischemia that reduce ATP concentrations or more specifically the ATP/ADP ratio (18). There are several known activators of the K_{ATP} channel, which include opioids, mild acidosis, acetylcholine, adenosine, and several other G protein coupled processes (23). Opioids have been suggested as being capable of activating this channel through coupling with the opioid receptor, perhaps making the channel less sensitive to ATP during ischemia (14, 20). We believe that activation of this channel might lead to hyperpolorization of the SA node to augment vagally mediated bradycardia during direct vagal stimulation (Appendix 108). However, based on the vagolytic effect of administered exogenous MEAP, potassium channel activation may also hyperpolarize the vagal nerve terminal and inhibit transmission via hyperpolorization of the nerve terminal (Appendix 108).

Summary

MEAP is one of the most abundant endogenous opiate peptides produced by the heart. Attempts to evaluate changes in the local myocardial enkephalins are complicated by rapid enkephalin degradation and dilution in route to the circulation where they are typically sampled. Despite heroic efforts to prevent degradation, enkephalins measured in the circulation probably represent a very small fraction of those actually present. However, measurable increases in endogenous MEAP were observed during the circulatory stress of hemorrhagic hypotension. When we infused MEAP systemically at picomolar rates (10-12pM), the critically important vagal control of heart rate was nearly abolished (9). Therefore, local myocardial MEAP is well positioned to serve as a paracrine regulator given its ability to inhibit vagally mediated bradycardia.

We recently reported that MEAP's vagolytic effect was produced in the local environment of the sinoatrial node, since MEAP directly administered to the sinoatrial node via microdialysis was able to inhibit vagally mediated bradycardia. These findings led us to propose the following **two hypotheses: 1) exogenous MEAP inhibited vagally mediated bradycardia through a delta opioid receptor mechanism, and 2) endogenous MEAP would increase during nodal artery occlusion and alter vagal function in a similar opiate receptor dependent fashion.**

Specific Aims

Specific Aim 1 was designed to determine the effective dose of nodal MEAP needed to regulate vagal bradycardia. Previous studies in our lab had shown that nodally administered MEAP (1mM) was capable of inhibiting vagally mediated bradycardia. The present study determined the maximally effective range of exogenously administered MEAP and determined the ED_{50}. The dose response of MEAP served as a baseline for comparison of opioid agonists and antagonists in subsequent studies. The MEAP dose response also served as a measure for comparison with endogenously produced MEAP in subsequent studies. The present study could facilitate the development of new therapeutic strategies to combat the adverse effects of autonomic imbalances.

Specific Aim 2 was designed to determine if inhibition of vagally mediated bradycardia by MEAP was elicited through a delta opioid receptor. Preliminary studies in our lab had shown that diprenorphine, a non-selective opioid antagonist, (100mM) was effective at blocking the inhibitory effects of MEAP on vagally mediated reductions in heart rate. The present study employed selective opiate receptor agonists and antagonists to 1) show that the MEAP effect was mediated at an opioid receptor, and 2) show which opioid receptor was responsible. MEAP had been shown to be a potent inhibitor of vagal control of heart rate. Inhibition of the normal parasympathetic control of resting heart rate has the potential to produce arrhythmias. Therefore, a selective delta receptor agent could be a beneficial antiarrhythmic agent.

Specific Aim 3 determined the effects of increasing endogenous MEAP on vagally mediated changes in heart rate. Previous studies had shown that hypotension was an effective stimulus to increase plasma enkephalin concentrations (36). Activation of the delta opioid receptor had been implicated in the beneficial effects of ischemic preconditioning (12, 49). Given that enkephalins were known to inhibit vagal function, this investigation allowed us to determine if 1) autonomic function was impaired during ischemia due to increased enkephalins, and 2) if so, was this impaired autonomic function correlated with MEAP acting on delta opioid receptors at the sinoatrial node.

Leu-arg, an inhibitor of enzymatic MEAP breakdown, was added to the perfusate to improve the concentration and recovery of endogenously produced MEAP. Tissue and circulating enzymes aggressively destroy MEAP. If the breakdown of MEAP was inhibited, then the local concentrations within the sinoatrial node should have increased.

Specific Aim 4 was designed to determine if increasing endogenous MEAP altered vagal bradycardia through delta opioid receptor activation of a K_{ATP} channel mechanism. Previous research had indicated that preconditioning effects were elicited through K_{ATP} channels. Blockade of the ATP sensitive K+ channels were shown by Gross et al (18) to prevent myocardial preconditioning in dogs. Opioid peptides were

capable of activating K_{ATP} channels within the heart and this activation was reversed by infusion of selective delta receptor antagonist, naltrindole. Naltrindole and glibenclamide were infused into the sinoatrial node via microdialysis. This line of investigation allowed us to examine the effects of endogenous opioids on vagal function and to determine if opioid effects were mediated through K_{ATP} channel activation by delta receptors in the sinoatrial node.

Significance

This research study could lead to treatments for increasing the salvage of myocardial tissue in patients who suffer heart attacks, and to the development of new strategies to combat the ever-present threat of reperfusion arrhythmias following myocardial ischemia. Studies done on patients, who suffer from complications due to congestive heart failure, have found that there is an autonomic imbalance present in these patients (4). As stated previously, patients who regain the normal balance between the parasympathetic and the sympathetic nervous systems were 3-5 times more likely to survive. The sympathetic drive, in patients with heart disease, was increased perhaps due to the immediate need to increase blood flow to areas of the heart with inadequate perfusion (15). This enhanced sympathetic drive was observed in heart failure patients even at rest, when the vagus nerve is normally the major controller of resting heart rate. This autonomic imbalance may cause arrhythmias (3, 4). Binkley et. al., in recent studies, have suggested that part of the therapeutic effect of angiotensin-converting enzyme inhibition in congestive heart failure patients

(improved survival) may be attributed to augmented vagal function (3). Therefore, the development of opiate receptor agents (agonists and antagonists) that can augment vagal function may prove antiarrhythmic if they restore autonomic balance (Appendix 112).

These studies may also lead to new insights into the interactions of enkephalins within the local nodal environment and enkephalin influences on the cardiac rhythm, during local nodal ischemia. These insights could lead to the identification of new strategies and therapeutic regimes to improve the survival of the post-ischemic heart.

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Chapter II

Delta Opioid Receptors Inhibit Vagal Bradycardia in the Sinoatrial Node

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ABSTRACT:

Background: Methione-enkephalin-arginine-phenylalanine (MEAP) is an endogenous opiate derived from the C-terminal sequence of the larger precursor molecule proenkephalin. This heptapeptide is abundant in the myocardium and has significant vagolytic activity when infused systemically. MEAP interrupted vagal bradycardia when it was delivered directly into the SA node by local microdialysis. This study was conducted to determine the opioid receptor responsible for MEAP's vagolytic effects. **Methods and Results:** Microdialysis probes were placed in the SA node of mongrel dogs and perfused at 5 microliters per minute. Increasing doses of MEAP were included in the nodal perfusate and approximately two-thirds of the vagal bradycardia was inhibited with a maximal effect at 0.3nmoles/μl and a half-maximal response near 0.1nmoles/μl. When deltorphin II (a delta opioid receptor agonist) was infused into the sinoatrial node more than 95% of the vagal bradycardia was eliminated at 0.3nmoles/μl with the half-maximal response near 0.1nmoles/μl indicating that deltorphin II was more efficacious than MEAP. The maximal deltorphin II and MEAP effects were both similarly reversed by the paired infusion of increasing doses of the delta opiate receptor antagonist, naltrindole. Selected mu- and kappa-receptor agonists (endomorphin, super DALDA, and dynorphin,U50 488) and antagonists (CTAP and nor BNI) were completely ineffective in this system. **Conclusions:** These data suggest that the vagolytic effect of MEAP involves the activation of delta opiate receptors within the SA node.

Keywords: Sinoatrial node, microdialysis, MEAP, vagolytic

INTRODUCTION

Endogenous opioids are a group of peptides, which have been implicated as neuromodulators in a number of biological systems (1, 2). The classical effects of opiates as analgesics have perhaps overshadowed many other important activities of these peptides. The study that follow illustrates that endogenous opioid peptides, such as methionine-enkephalin-arginine-phenylalanine (MEAP), can play a crucial role in the regulation of the cardiovascular system.

MEAP is one of the most abundant opiate peptides in the heart. MEAP is efficiently elaborated as one of several constituent enkephalins synthesized as part of the larger precursor molecule, proenkephalin (3). Attempts to evaluate changes in the local myocardial enkephalins are complicated by rapid enkephalin degradation and dilution in route to the circulation where they are typically sampled. Despite heroic efforts to prevent degradation, enkephalins measured in the circulation probably represent a very small fraction of those present in the tissue of origin. Increases in endogenous MEAP have been reported during hemorrhagic hypotension and ischemia. When we infuse MEAP systemically at picomolar rates, the critically important vagal control of heart rate is nearly abolished (4, 5). Therefore, MEAP is well positioned to serve as a paracrine regulator of local heart rate control.

Since both the intracardiac parasympathetic ganglia and SA node are anatomically adjacent and they share the same blood supply, determining the precise location of MEAP's vagolytic effect has been particularly difficult. For the first time ever, we successfully placed miniature microdialysis probes within the substance of

the cardiac pacemaker (SA node) in *vivo*. The probe allowed us to precisely regulate nodal function and to sample the previously inaccessible paracrine environment of the working node. In most instances, placement of the probes in the SA node was achieved without noticeable impairment of nodal function or its neural control. Dialysate perfusion of the SA node with micromolar amounts of norepinephrine produced a significant tachycardia. Direct stimulation of the sympathetic nerve continued to produce a significant increase in heart rate and direct vagal stimulation produced a significant bradycardia. Using microdialysis, we have collected data, which suggest that, the "vagolytic" effect of MEAP results from the local nodal inhibition of parasympathetic neurotransmission, probably by reducing acetylcholine release from vagal nerve fibers in the SA node (6). Microdialysis will allow us to maintain the sinoatrial node and its innervation functionally intact while we carefully dissect and isolate the complex local neuroendocrine and paracrine environment, which regulates it. The series of experiments which follows were specifically designed to 1) determine the concentration of MEAP required to produce functional effects on vagal control of heart rate, and 2) determine which receptors are responsible for MEAP's vagolytic effects.

METHODS

Surgical Preparation

Sixty mongrel dogs of either gender weighing (15-25kg) were assigned at random to various experimental protocols. All protocols were approved by the Institutional Animal Care and Use Committee and were in compliance with the *NIH Guide for the Care and Use of Laboratory Animals*. The animals were anesthetized with sodium pentobarbital (32.5mg/kg), intubated and mechanically ventilated initially at 225mls/min/kg with room air. Fluid filled catheters were then inserted into the right femoral artery and vein and advanced into the descending aorta and inferior vena cava, respectively. The arterial line was attached to a Statham PD23XL pressure transducer and heart rates and arterial pressure were measured continuously on-line (MacLabs). The venous line was used to administer supplemental anesthetic, as required. The acid-base balance and the blood gases were determined with an Instrumental Laboratories Blood Gas Analyzer. The pO2 (90-120mmHg), the pH (7.35-7.45) and the pCO2 (30-40mmHg) were adjusted to normal by administering supplemental oxygen, bicarbonate or modifying the minute volume.

The right and left cervical vagus nerves were isolated through a ventral midline surgical incision. They were ligated with umbilical tape and replaced for later retrieval. Surgical anesthesia was carefully monitored, and a single dose of succinylcholine (50μg/kg) was administered intravenously to temporarily reduce involuntary muscle movements during the 10-15minutes required for electrosurgical incision of the chest and removal of the ribs 2-5. The heart was exposed from the

right aspect. The pericardium was opened and the dorsal pericardial margins were sutured to the body wall to provide a pericardial cradle.

Nodal Microdialysis

A 25g stainless steel spinal needle containing the microdialysis line was inserted into the center of the long axis of the sinoatrial node. The needle was removed and the probe was then positioned so that the dialysate window was completely in the substance of the sinoatrial node. The microdialysis probes (Fig 1), were constructed of a single 1cm length of dialysis fiber from a Clirans 10 (Terumo Medical Corporation, Sumerset, NJ) artificial kidney (7). The dialysis tubing had a 300μm ID, a 305μm OD and a molecular weight cutoff of 5,000 KD. The inflow and outflow lines were constructed of hollow 170μm OD silica tubing (SGE, Austin, TX) glued into the dialysis fiber.

Evaluation of the Microdialysis Probe

Norepinephrine (1nmol/μl) was diluted in 0.1% ascorbic acid in 0.9% isotonic saline (vehicle) and perfused into the microdialysis probe at a rate of 5μl/min with the aid of a microinfusion (BAS) pump to confirm functionally the position of the probe in the SA node. If the probe was properly positioned, the heart rate routinely increased by 30-40 bpm within 30 seconds of introducing the norepinephrine. This tachycardia was not observed when the probe was not optimally placed in the SA node. The norepinephrine was immediately flushed out, and the system was perfused with saline (5μl/min) for 1 hour and permitted to reequilibrate before beginning the experimental protocol. At the conclusion of nine experiments, the probe was removed

and repositioned parallel to but 1-2mm distant from the original insertion. No significant changes in heart rate were observed at the new probe position, when norepinephrine was reintroduced. In addition, preliminary unpublished micrography of the SA node tissue around the probe is consistent with expected nodal histological organization (27, 32).

Protocol I: ***Determining the effective vagolytic concentration of exogenous MEAP*** (Appendix 106).

Previous findings have indicated that the process of inserting the probe is accompanied by an increase in adenosine and norepinephrine in the immediate area of the probe (7). After an hour, these molecules were measured to return to low baseline concentration (7). Therefore, the microdialysis probe was perfused with isotonic saline for a period of one-hour to allow for a reestablishment of baseline levels for all components in the nodal environment. After this period, control vagal responses were obtained by electrically stimulating the right vagus nerve at 1, 2 and 3 hertz. The vagus was stimulated at a supra-maximal voltage for 15 seconds each and 2 minutes were allowed between stimuli to permit the vagus to recover. Various doses of MEAP (0.01mM, 0.03mM, 0.1mM, 0.3mM and 1.0mM) were infused for 5 minutes, before stimulating the vagus, as previously described at 1, 2, and 3Hz. Between each subsequent administration of enkephalin, isotonic saline was infused into the probe and the vagus was retested to ensure that function had returned to normal and that the peptide had washed out.

Protocol II: ***Determination of an opioid receptor subtype responsible for MEAP's vagolytic effects*** (Appendix 106, 107).

Microdialysis probes were inserted and as described above control vagal responses were obtained. MEAP inhibition of vagally mediated bradycardia was established through infusion of the maximally effective concentration of MEAP (0.3mM) into the probe. Once blockade was established, progressive inhibition of the MEAP effect was elicited through coinfusion of increasing doses of naltrindole, a selective delta antagonist, (0.001mM, 0.02mM, 0.07mM, and 0.22mM). The vagus was stimulated at 1, 2 and 3 hertz 5min after infusion of each naltrindole concentration. Similar dose response curves were constructed by coinfusing MEAP with selective mu (CTAP) and kappa (nor-Binaltorphimine) antagonists.

Protocol III: ***Confirmation of the opioid receptor subtype responsible for MEAP's vagolytic effect*** (Appendix 106).

The previously described protocol for probe insertion and obtaining control vagal responses were again employed. Various doses of a selective delta receptor agonist, deltorphin II (0.01, 0.03, 0.1, 0.3, 1mM), was infused into the microdialysis probe for a period of 5 minutes. The vagus was stimulated at 1, 2, and 3Hz during each deltorphin infusion. In four additional experimental groups selective mu (endomorphin I, and Super DALDA) and kappa (U50488, and dynorphin) agonists were infused into the probe. All agonists were infused at similar doses to deltorphin II

and vagal function was reevaluated during each subsequent dose of selective mu or kappa agonist.

MATERIALS:

MEAP, and Deltorphin II were purchased from the American Peptide Co, in Sunnyvale, CA. Naltrindole, nor-Binaltorphimine, U50, 488, dynorphin, enomorphin I and CTAP were purchased from Sigma RBI in St. Louis, MO. Super Dalda was a gift from Drs. Hazel Szeto and Peter Schiller. Microdialysis probes were fabricated by Dr. David G. L. Van Wylen of St. Olaf College in St. Olaf, Minnesota.

DATA ANALYSISA:

The data were expressed as mean ± standard error. Differences were evaluated by analysis of variance and multiple post-hoc comparisons were made with Tukey's post-hoc test (GB-STAT^{TM}, Dynamic Microsystems, Silver Spring, MD). Repeated measures designs were used where appropriate. $P < 0.05$ was accepted as a statistically significant difference.

RESULTS:

Sixty animals were divided into nine groups of 6-7 animals each. Of these, fifty-seven animals completed their experimental protocols. Three animals were excluded from the final analysis in the absence of acceptable baseline vagal responses. Initial control responses were similar for all nine groups studied. The resting heart rate was unaltered by any of the six opioids or three opiate receptor antagonists added into to the dialysis line perfusing the SA node regardless of dose. MEAP reduced vagally mediated bradycardia by 60-70% with an apparent threshold effect at a perfusate concentration of 0.03mM (Figure 1). The dose response was nearly identical at each of the three stimulation frequencies. The maximal effect was observed at 0.3mM with an apparent EC50 approximating 0.1mM. After each dose, the peptide was washed out and vagal response returned to baseline within 5 minutes.

In the next group MEAP (0.3mM) was introduced into the dialysate and the maximum vagolytic effect observed above was verified (Figure 2). The delta opiate receptor antagonist, naltrindole was then added to the nodal perfusate alone and in combination with the maximally effective concentration of MEAP. Naltrindole had no effect of its own but completely reversed the vagolytic effect of MEAP and restored vagal control of heart rate to baseline (Figure 2). The dose response for reversal was very narrow (Figure 3). There was an apparent threshold for naltrindole near 0.02mM with the reversal of the vagolytic effect being complete by 0.07mM. The IC_{50} was estimated at 0.03mM.

Equimolar dose response relationships were constructed with the mu-receptor antagonist, CTAP, and the kappa-receptor antagonist, norBNI. As with naltrindole, the resting heart rate was unaltered. However in contrast to naltrindole, neither agent had any influence on the vagolytic effect of MEAP. The dose responses are not included but the effect of the highest dose of each is illustrated in Figure 4 for direct comparison with naltrindole. These data strongly suggest that the vagolytic effect of MEAP results from an interaction with a delta opiate receptor within the SA node.

Next, dose response curves were constructed with receptor subtype specific agonists. The delta specific agonist, deltorphin II, was added to the dialysis line in concentrations similar to those used for MEAP. Deltorphin did not alter the resting heart rate. However, deltorphin produced a dose dependent inhibition of the vagally mediated bradycardia similar to but not identical to the effect of MEAP. The normalized dose response curves were nearly superimposable and much like MEAP, there was an apparent threshold effect at 0.03 mM with a subsequent maximal inhibition at 0.3mM (Figure 5). The maximal effect of deltorphin (95%) was however consistently greater than that observed with MEAP (67%) (Figure 6). Like MEAP, the response to deltorphin was completely reversed by naltrindole in a dose relationship identical with that observed versus MEAP. This confirms delta receptor participation in the response but the greater efficacy of deltorphin suggests that the response to MEAP may be more complex.

Additional dose responses were constructed with mu– and kappa-receptor selective agonists to further rule out other non-delta opiate receptor participation in

the vagal inhibition. The endogenous mu-agonist, endomorphin and the highly selective synthetic mu-agonist, super DALDA, were evaluated to determine if mu-receptors were involved. Neither of these agents had any effect on resting heart rate or on the vagal bradycardia observed during vagal nerve stimulation (Figure 7). To test for participating kappa-receptors, the endogenous kappa-agonist dynorphin 1-13 and the selective synthetic kappa-agonist, U50488 were administered in increasing doses. Once again, the results were the same. Neither kappa-agent modified resting heart rate or the subsequent vagally mediated bradycardia (Figure 8). These data confirm the delta receptor character of the vagolytic response.

DISCUSSION:

The aim of the current studies was to determine the opiate receptor subtype through which MEAP exerted its vagolytic activity. This was accomplished by creating a profile of the responses with a large group of subtype selective agonists and antagonists. This goal arose as a result of earlier studies that established that systemic MEAP was vagolytic (4, 5) and that it regulates heart rate primarily via opiate receptors localized in the SA node (6). Before direct comparisons could be made with the larger group of more selective agents, a dose effect relationship for the nodal delivery of MEAP had to be determined. This dose effect relationship provided a threshold response near 0.03mM, a maximal response at 0.3mM and an EC_{50} near 0.1mM. Based on binding studies conducted with dog brain membranes the IC50 for MEAP estimated in vivo is significantly higher than might be expected (8). This apparent discrepancy could result from a very inefficient transfer of peptide between the perfusate and the surrounding interstitial fluid. This observation is a reminder that although one can estimate accurately perfusate/dialysate transfer rates in vitro, it is often much more difficult and largely impractical to estimate the rate of transfer into the semi-solid interstitial environment surrounding the dialysis probe in vivo. In addition to the colloidal impediment to diffusion, other factors such as charge, adsorption and local metabolism can increase the discrepancy between the concentrations inside and outside the dialysis probe. The nanomolar KDs that have been reported for most opiate receptors suggest that the transfer into the tissue is likely to be less than 1%. Preliminary results with radiolabeled peptide suggest a

reasonable transfer of approximately 20-30%. However, these same data indicate the resulting steady state tissue concentration is much lower yet; in the low nanomolar range suggesting that dispersal or destruction of the peptide is faster than its rate of transfer into the tissue. The estimated nanomolar concentration would be more consistent with known receptor interactions. A variety of molecules (native and synthetic) were used for the subsequent agonist/antagonists comparisons in order to minimize the possibility that differences in effect were the result of differences in permeation or metabolism.

In order to determine the opiate receptor subtype with which MEAP interacts, more selective tools had to be employed. Prior studies had not provided any indication of receptor subtype because they used the high affinity but non-selective opiate antagonist, diprenorphine. The determination of subtype is complicated further because MEAP is relatively promiscuous and binds well to all three (μ, δ & κ) receptor subtypes. When the delta-receptor antagonist, naltrindole was introduced into the perfusate, it reversed the vagolytic effect of MEAP in a dose dependent fashion. The delta-receptor selectivity of the reversal is supported by an IC_{50} near 30μM well below the competing agonist concentration of 300μM.

The delta-selective agonist, deltorphin II, largely duplicated the vagolytic effect of MEAP. The effective dose response was nearly identical and the effect of deltorphin displayed a similar EC_{50}, very near 100μM. Further support for the delta receptor hypothesis was obtained with naltrindole, which completely reversed the vagolytic effect of deltorphin in the same concentration range that was effective

versus MEAP. One difference observed was that deltorphin produced a greater maximal effect than MEAP. With deltorphin, the interruption of vagal bradycardia was nearly complete while the maximal effect observed with MEAP was 60-70% inhibition. This suggested that either deltorphin has a greater intrinsic effect or the more promiscuous MEAP was activating an opposing opiate receptor and thus moderates its own effect. When other selective agonists were employed no vagolytic activity could be demonstrated with mu-selective (endomorphin, super Dalda) or kappa-selective (dynorphin 1-13, U50488) agonists. When viewed together with the deltorphin results, the exclusive delta character of the vagolytic effect is clearly apparent.

Although the greater efficacy of deltorphin may have arisen from a greater intrinsic activity, it may also reflect a lower steady state concentration for MEAP secondary to a greater clearance or a greater rate of local metabolism. Consistent with the theory of differential metabolism, one would expect that deltorphin's native D-alanyl residue would render it more resistant to the ubiquitous N-terminal aminopeptidases. The similar plateau observed in the two dose responses tends to rule out metabolism or clearance as an explanation since higher doses should overcome either of these factors assuming the conductance capabilities of the dialysis probes have not been exceeded.

The absence of participation by mu- and kappa-receptors in the effect of MEAP was verified by adding selective mu- and kappa-antagonists into the nodal perfusate. As indicated neither of these agents were able to modify the vagolytic

effect of MEAP. These findings provide additional support for MEAP exerting its vagolytic effect almost entirely through a delta-receptor mechanism. The absence of a deltorphin-like increase in efficacy in the presence of these antagonists argues against the hypothesis described above, that MEAP interacts with an opposing (vagotonic) opiate receptor which then limits its simultaneous vagolytic activity.

When considered in sum, these data firmly support the presence of a population of nodal delta-opiate receptors that are highly vagolytic and presumably located on parasympathetic nerve terminals. Prior studies have localized the receptors responsible for this effect exclusively to the SA node (6). The prejunctional location of the receptors was proposed because MEAP was totally ineffective versus the direct acting muscarinic agonist, methylcholine (4). The circumstances under which these receptors operate either physiologically or pathologically remain to be determined. However, exercise training is characterized by improved vagal function and hypertension and heart failure are accompanied by impaired vagal function. Might these functional differences in vagal status result in part from alterations in the nodal opiates and/or their receptors? Nodal opioids are increased during ischemia (9) and delta-opioids have been implicated in ischemic preconditioning (10, 11). How important circulating opioids are in these processes is unclear, but the heart clearly makes opioids (12) and as such these local hormones may represent an important class of paracrine regulators in health and disease.

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LEGENDS:

Figure 1. This figure provides a graphical representation of the dose response relationship between MEAP and vagal inhibition of bradycardia (n = 12).

Figure 2. This figure illustrates the effect of MEAP and the delta-opioid receptor antagonist, naltrindole (NT) on heart rate/frequency response relationships during right vagal stimulation. In this example, the concentrations of MEAP and naltrindole were 0.3 mM and 0.22 mM respectively. The (*) indicates that MEAP is significantly different from the other three treatment groups (n = 7).

Figure 3. This figure provides a graphical representation of the dose response relationship for the ability of naltrindole to reverse the inhibition of vagally mediated bradycardia by MEAP (n = 6).

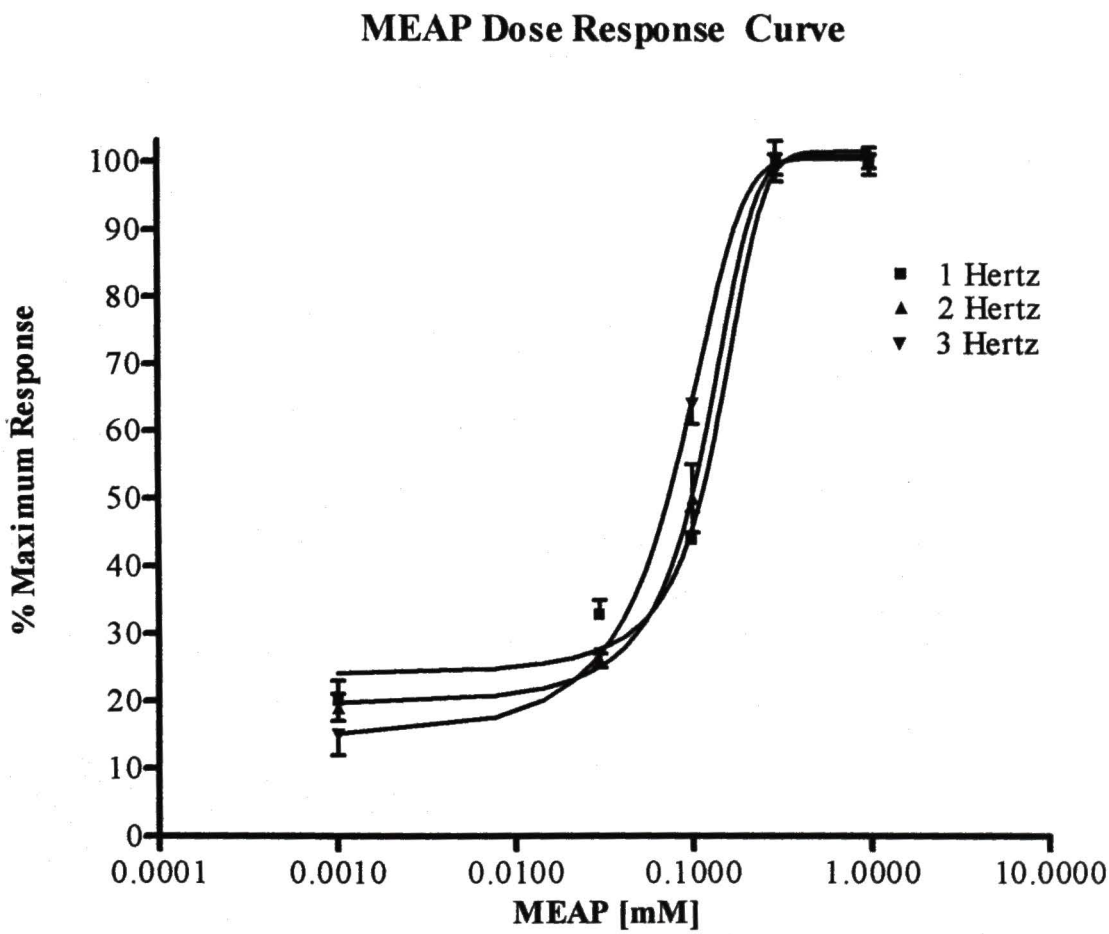
Figure 4 This figure illustrates the inability of nodal mu (CTAP) and kappa (nor-BNI) opioid receptor antagonists to reverse the inhibitory effect of MEAP during right vagal stimulation. The antagonist only responses were not different from vehicle (saline) and were omitted for clarity. In this example, the concentration of MEAP was 0.3 mM and that of the antagonists was 0.22 mM. The (*) indicates responses significantly different from vehicle or MEAP + naltrindole (NT) (CTAP, n = 6; norBNI, n = 6). [nor-BNI (norbinaltorphimine); CTAP (D-Phe-Cys-Tyr-D-Trp-Ala-Thr-Pen-Thr-NH_2)]

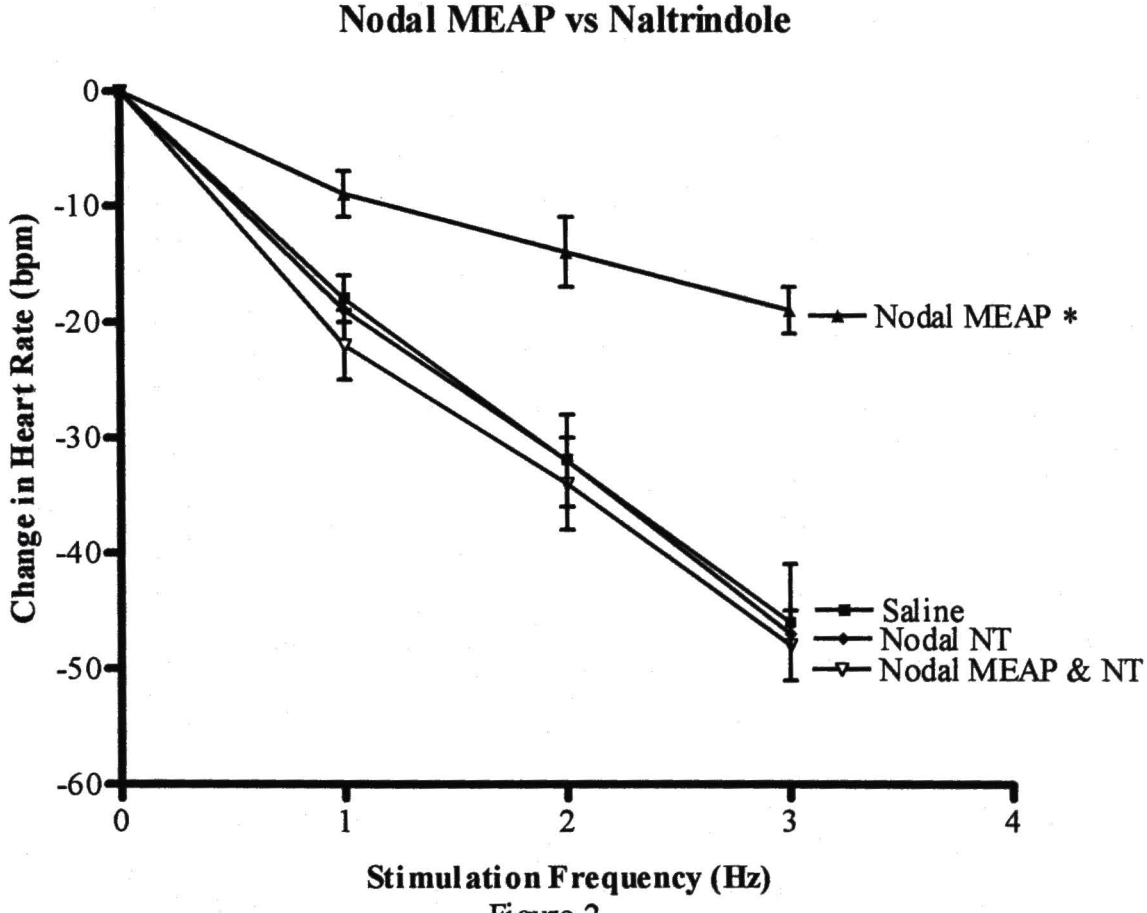
Figure 5. This figure provides a graphical representation of the dose response relationship between, a delta agonist, deltorphin II and the inhibition of vagally mediated bradycardia (n = 7).

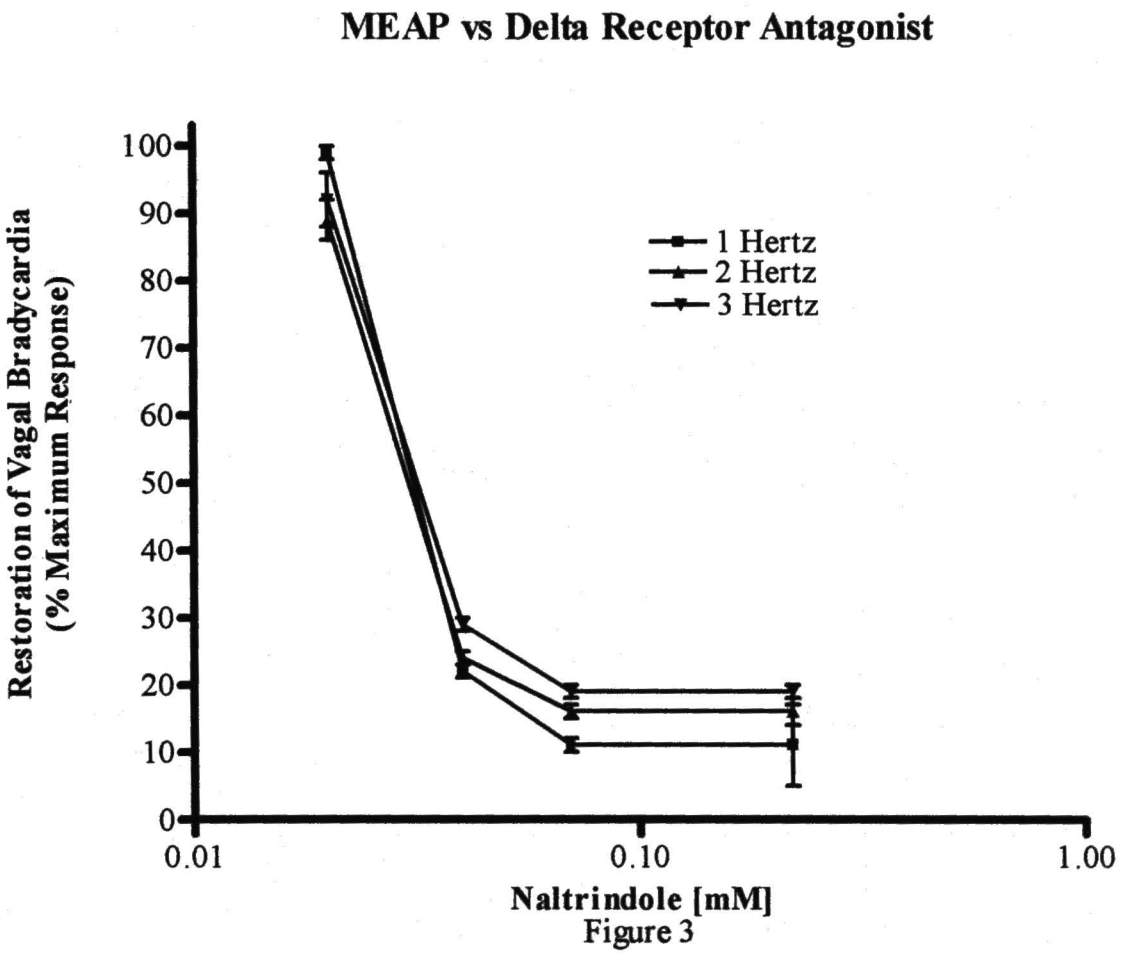
Figure 6. This figure illustrates the effect of deltorphin II (Del II) and the delta-opioid receptor antagonist, naltrindole (NT) on heart rate/frequency response relationships during right vagal stimulation. In this example, the concentrations of deltorphin and naltrindole were 0.3 mM and 0.22 mM respectively. The (*) indicates that deltorphin is significantly different from the other three treatment groups (n = 7).

Figure 7. This figure illustrates the failure of two mu selective opioids to alter the heart rate/frequency response relationships during right vagal stimulation. In this example, the concentrations of endomorphin and super DALDA (Tyr-D-Arg-Phe-Lys-NH2) were 0.3 mM. The effect of MEAP is included for comparison (n = 7).

Figure 8. This figure illustrates the failure of two kappa selective opioids to alter the heart rate/frequency response relationships during right vagal stimulation. In this example, the concentrations of dynorphin 1-13 and U50488 were 0.3 mM. The effect of MEAP is included for comparison (n = 7).


MEAP Dose Response Curve
% Maximum Response
100
90
80
70
60
50
40
30
20
10
0
0.0001
0.0010
0.0100
0.1000
1.0000
10.0000
MEAP [mM]
1 Hertz
2 Hertz
3 Hertz
Figure 1


Nodal MEAP vs Naltrindole
Change in Heart Rate (bpm)
0
-10
-20
-30
-40
-50
-60
0
1
2
3
4
Stimulation Frequency (Hz)
Nodal MEAP *
Saline
Nodal NT
Nodal MEAP & NT
Figure 2


MEAP vs Delta Receptor Antagonist
Restoration of Vagal Bradycardia
(% Maximum Response)
100
90
80
70
60
50
40
30
20
10
0
0.01
0.10
1.00
1 Hertz
2 Hertz
3 Hertz
Naltrindole [mM]
Figure 3

Delta Receptor Antagonist Inhibits MEAP's Vagolytic Effects

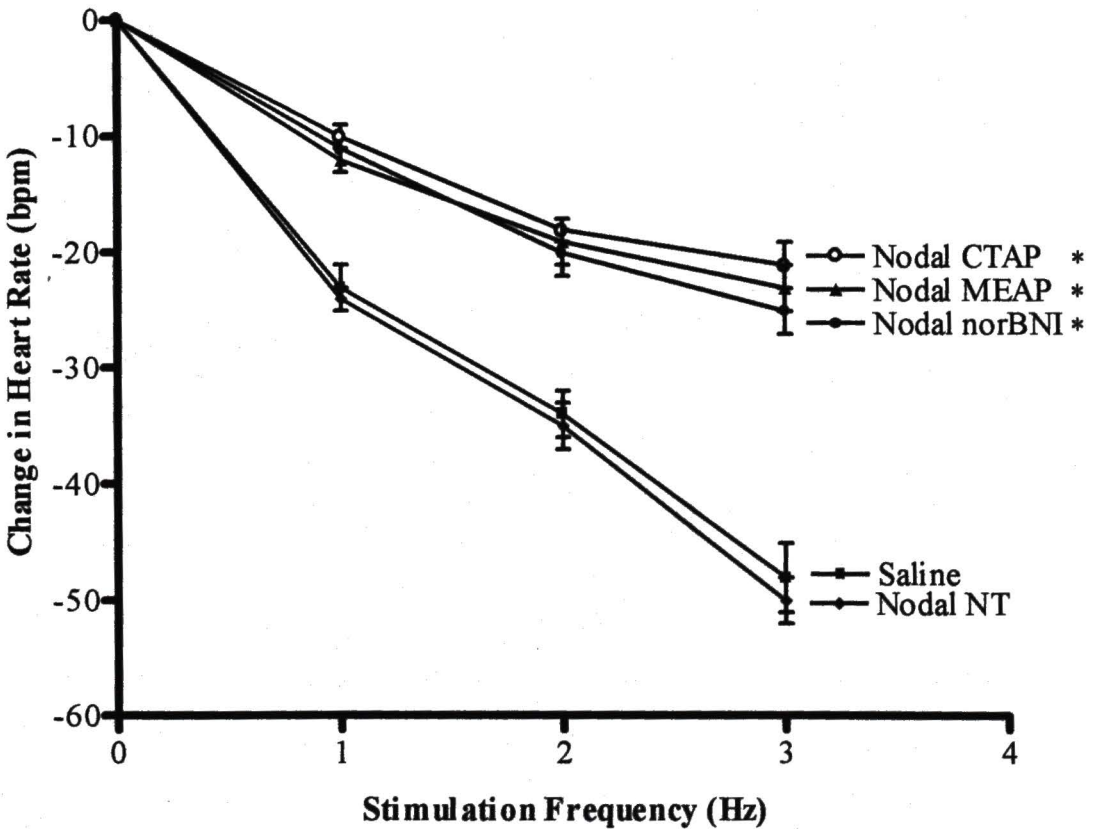


Figure 4

Deltorphin II Dose Response Curve

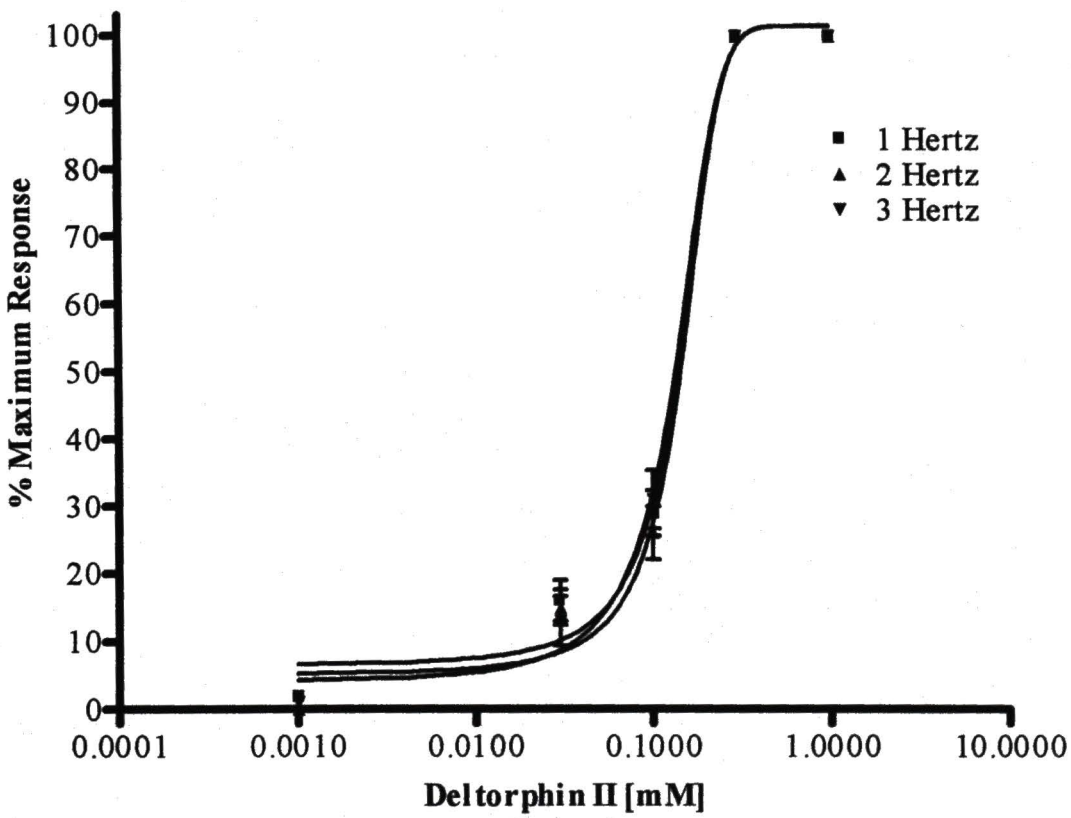


Figure 5

Selective Delta Agonist is MEAP is More Effective than MEAP

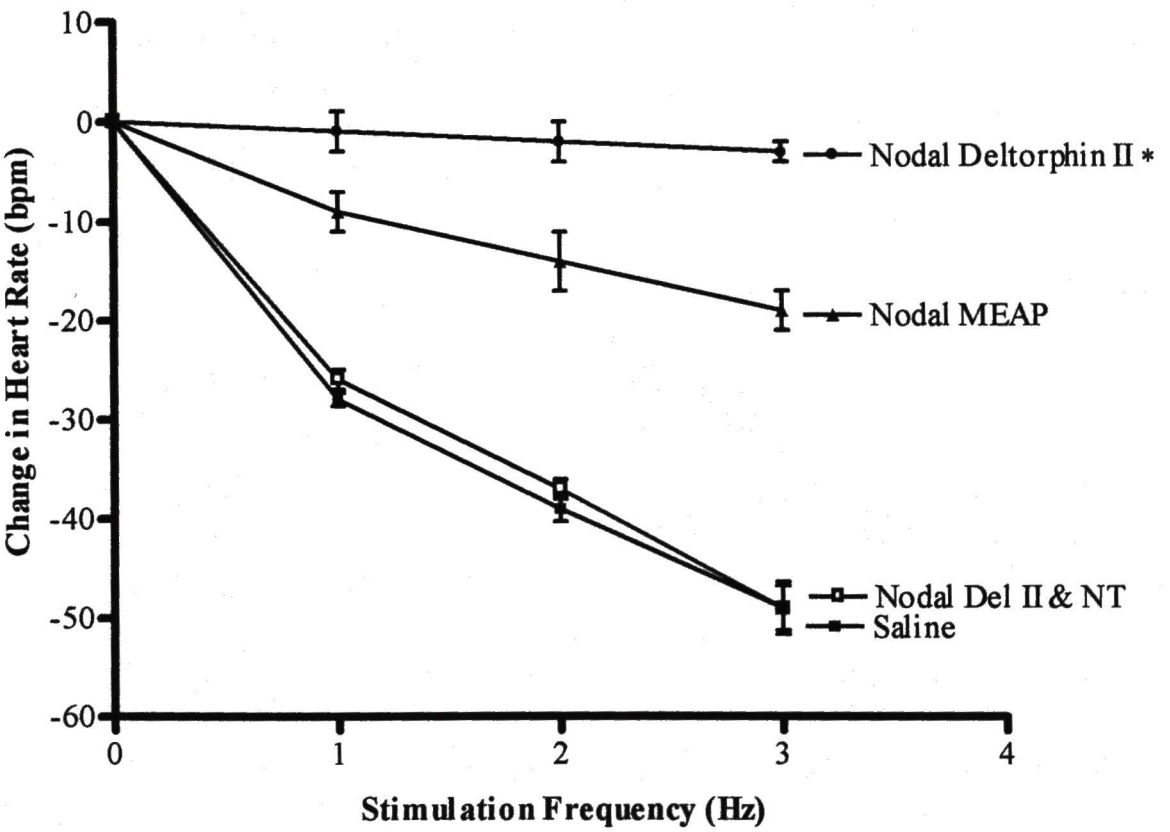


Figure 6

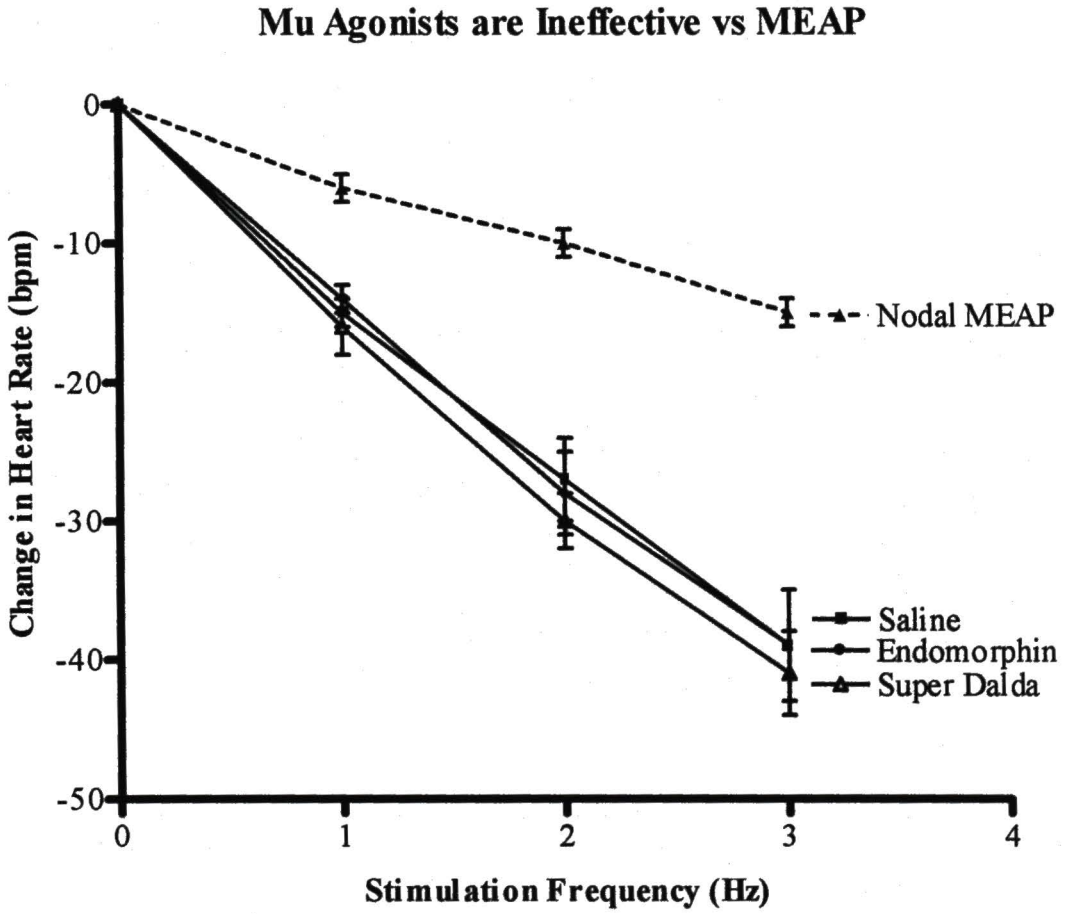
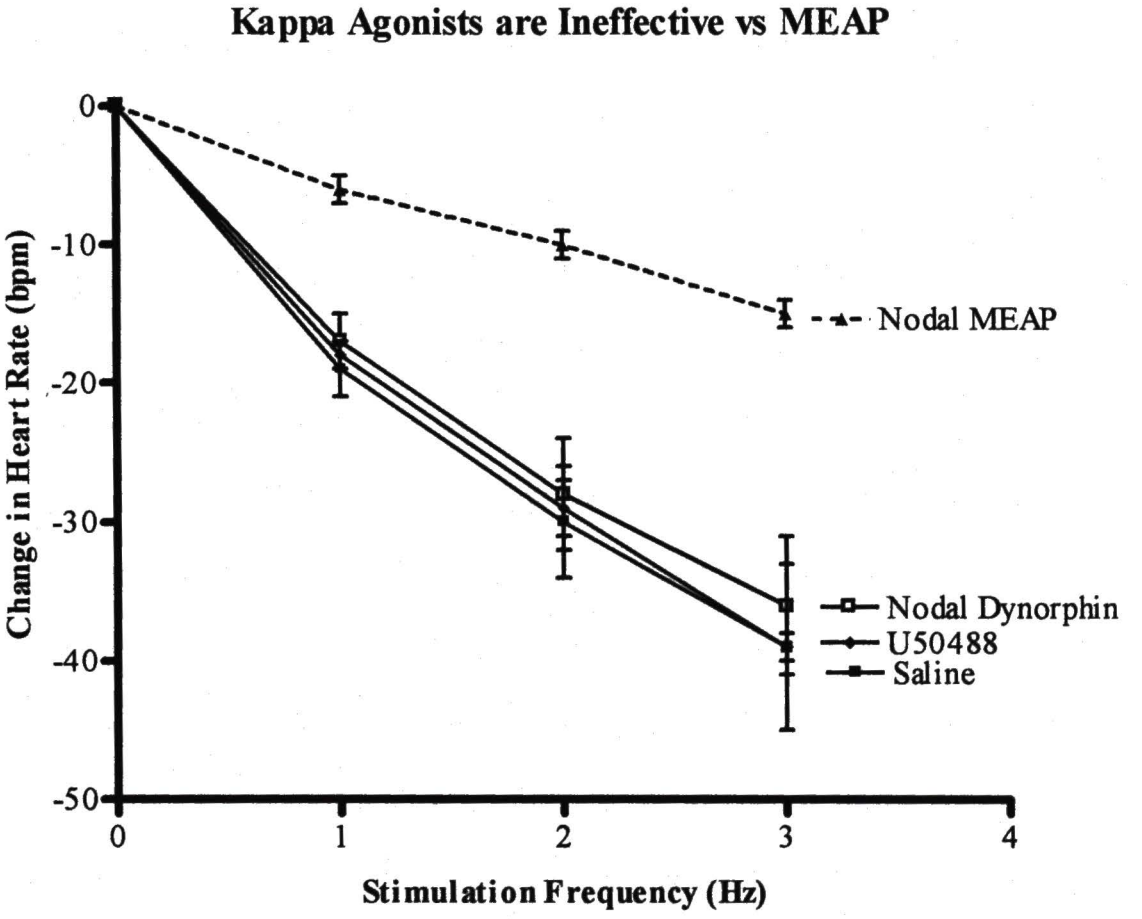

Mu Agonists are Ineffective vs MEAP
Change in Heart Rate (bpm)
0
-10
-20
-30
-40
-50
0
1
2
3
4
Stimulation Frequency (Hz)
Nodal MEAP
Saline
Endomorphin
Super Dalda

Figure 7


Kappa Agonists are Ineffective vs MEAP
0
-10
-20
-30
-40
-50
Change in Heart Rate (bpm)
0
1
2
3
4
Stimulation Frequency (Hz)
Nodal MEAP
Nodal Dynorphin
U50488
Saline
Figure 8

PREFACE TO CHAPTER III

The opiate receptor data clearly indicate that the nodal administration of MEAP produces it's vagolytic effect through a delta receptor mechanism. This then leads to the second question, does endogenous MEAP produce a qualitatively and quantitatively similar effect. This chapter describes the development of the microdialysis technique and it's application to recover endogenously produced MEAP. For the first time, we have been able to recover and measure nodal MEAP produced during local reduction in nodal blood flow. Given that our previous data demonstrated that MEAP was vagolytic when delivered to the sinoatrial node, we wanted to evaluate the effects of endogenously produced MEAP. We hypothesized that there would be an increase in enkephalin in the node, as measured by a selective radioimmunoassay for MEAP. We also hypothesized that this increase in enkephalin would be correlated with an inhibition of the vagus, similar to observations during exogenous MEAP infusion. Given the previous findings that the vagolytic effects of MEAP were associated with a delta opioid receptor mechanism, we hypothesized that endogenous MEAP's vagolytic effects would also be associated with a delta opioid receptor mechanism. The most striking finding, during this series of experiments was that contrary to expectations, there was an enhanced vagal effect observed during reduced nodal blood flow. Therefore, we also evaluated for delta opioid receptor participation in the enhanced vagal effect observed during low flow through the node. Previous research has demonstrated that there was a cardioprotective effect during ischemia elicited by opioid interaction with a K_{ATP} channel. This channel can

become activated when the ADP/ATP ratio is reduced, such as during low blood flow. This led us to test the hypothesis that the enhanced vagal bradycardia, during low flow results from a delta opioid receptor activation of a K_{ATP} channel.

Chapter III

Transient Arterial Occlusion Raises Enkephalin in the Sinoatrial Node and Improves Vagal Bradycardia in Canines

First Author: Jackson
Short Title: Hypoperfusion, Enkephalin and Nodal Bradycardia

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ABSTRACT: The C-terminal proenkephalin sequence, Met-enkephalin-arg-phe (MEAP), is abundant in the myocardium and has significant vagolytic activity. The study that follows was conducted to determine if endogenous nodal MEAP increases during hypoperfusion of the sinoatrial node and does it alter vagal function. Microdialysis probes were placed in the canine SA node, perfused at 5 microliters per minute and the SA node artery was occluded and released intermittently (10min intervals). The intermittent artery occlusion was then followed by periods of prolonged reduced blood flow (30min). Vagally mediated bradycardia was compared before, during, and after arterial occlusion. An increase in MEAP release (0.07 – 0.22pmol) was recorded during each of the initial 10 minute occlusion periods. MEAP returned to baseline during each subsequent 10-minute reperfusion. There was a sustained increase in MEAP (0.11 - 0.15pmol) during longer occlusions. Surprisingly the increased MEAP during hypoperfusion was coincident with improved vagal bradycardia. The improved vagal function appears to involve an interaction between delta receptors and ATP-sensitive K^+ channels since the augmentation was blocked or reduced respectively when naltrindole or glibenclamide were included in the microdialysis probe. These data support the hypothesis that vagal function improves during reduced blood flow to the sinoatrial node due to an increase in opiate activity which interacts with local delta receptors and possible due to activation of nodal ATP-sensitive K^+ channels.

Keywords: Sinoatrial node, microdialysis, hypoperfusion

INTRODUCTION

The autonomous pacemaking of the heart resides in a small concentration of cells collectively identified as the Sinoatrial Node (SA node). This collection of cells is highly innervated by both arms of the autonomic nervous system, namely the parasympathetic (vagus) and sympathetic nerves. Normally, the beat to beat control of the heart rests almost exclusively with the vagus nerve, which serves as an ever-present brake on the heart. Instantaneous changes in heart rate are primarily achieved by increasing or withdrawing the vagal effect. The practical significance of this vagal influence is illustrated by the fact that patients who regain vagal control of heart rate soon after suffering heart attacks are far more likely to survive long term (Kleiger et al., 1987).

Prior studies have demonstrated a number of interactions between endogenous opiates and the cardiovascular system (Caffery, 1999; Little et al., 1998). The opioid peptides which were first recognized for their ability to modulate pain, are widely distributed in the peripheral autonomic nervous system, where they are well positioned to influence cardiovascular functions (Caffery, 1999; Musha et al., 1999). Although many opioid activities in the cardiovascular system are mediated by regulating neurotransmitter release, other actions may also include the post-junctional modification of cardiovascular targets. Enkephalin's inhibition of the vagally mediated responses such as increased coronary blood flow, decreased contractility and bradycardia are examples of the former (Caffery, 1999; Caffery et al., 1995). The ability of enkephalins to reduce the contractile response to norepinephrine in isolated

cardiac myocytes is an example of the latter (Xiao et al., 1997).

The normal function of the endogenous opiates within the cardiovascular system is not well understood despite several clear cardiovascular effects observed when opiate peptides are administered. Methionine-enkephalin-arginine-phenylalanine (MEAP), an endogenous opiate derived from the C-terminal sequence of proenkephalin (Younes et al. 2000), is one of the most abundant opiate peptides in the myocardium (Barron et al., 1992; Mateo et al., 1995). When administered systemically, MEAP immediately and reversibly blocks vagal control of heart rate, contractile activity and coronary blood flow (Caffery, 1999). When administered directly into the sinoatrial node, MEAP significantly inhibited vagally mediated bradycardia (Farias et al., 2001). Proenkephalin is produced in the heart (Howells et al., 1986; Lang et al., 1999; Springhorn et al., 1992) and MEAP increases in the circulation during circulatory stress (Mateo et al., 1995). As a result, MEAP may be an important myocardial regulator of vagal control of the heart.

In contrast, the cardiovascular consequences of altering endogenous opioid production are much less well defined. The specific circumstances in which endogenous circulating or cardiac opioids can be elevated acutely are also not well understood. Cardiac opioid content is elevated in neonatal and senescent animals (Boluyt et al., 1993; Caffrey et al., 1994). Higher concentrations of endogenous opioids have been associated with both hemorrhagic hypotension and ganglionic blockade (Bruckner et al., 1984; Mateo et al., 1995). Opioid systems have also been

implicated in cardiac preconditioning (Miki et al., 1998; Schultz et al., 1998; Schultz et al. 1997a; Schultz et al., 1997b).

This series of investigations was based on pervious work in our lab, which demonstrated that MEAP delivered directly into the sinoatrial node was significantly vagolytic (Farias et al., 2001). This study was undertaken to address the following questions: 1) are cardiac opioids released locally in the SA node, 2) do they modify vagal function, 3) do the observed effects involve opiate receptors and 4) which opioid receptor sub-type is likely involved.

MATERIALS AND METHODS:

Surgical Preparation

Twenty mongrel dogs of either gender weighing 15-25kg were assigned at random to various experimental protocols. All protocols were approved by the Institutional Animal Care and Use Committee and were in compliance with the *NIH Guide for the Care and Use of Laboratory Animals*. The animals were anesthetized with sodium pentobarbital (32.5mg/kg), intubated and mechanically ventilated initially at 225mls/min/kg with room air. Fluid filled catheters were then inserted into the right femoral artery and vein and advanced into the descending aorta and inferior vena cava, respectively. The arterial line was attached to a Statham PD23XL pressure transducer and heart rates and arterial pressure were measured continuously on-line (MacLabs). The venous line was used to administer supplemental anesthetic, as required. The acid-base balance and the blood gases were determined with an Instrumental Laboratories Blood Gas Analyzer. The pO2 (90-120mmHg), the pH (7.35-7.45) and the pCO2 (30-40mmHg) were adjusted to normal by administering supplemental oxygen, bicarbonate or modifying the minute volume.

The right and left cervical vagus nerves were isolated through a ventral midline surgical incision. They were ligated with umbilical tape to inhibit afferent nerve traffic and replaced for later retrieval. Surgical anesthesia was carefully monitored, and a single dose of succinylcholine (50μg/kg) was administered intravenously to temporarily reduce involuntary muscle movements during the 10-15 minutes required for electrosurgical incision of the chest and removal of the ribs 2-5.

The heart was exposed from the right aspect. The pericardium was opened and the dorsal pericardial margins were sutured to the body wall to provide a pericardial cradle.

A 25g stainless steel spinal needle containing the microdialysis line was inserted into the center of the sinoatrial node along its long axis. The needle was removed and the probe was then positioned so that the dialysis window was completely within the substance of the sinoatrial node (Fig. 1). The sinoatrial node can be identified in the canine heart as a faint white area located at the junction of the superior vena cava and the right atrium. The microdialysis probes were constructed of a single 1cm length of dialysis fiber from a Clirans 10 (Terumo Medical Corporation, Sumerset, NJ) artificial kidney (Van Wylen et al., 1990). The dialysis tubing had a 300μm ID, a 305μm OD and a molecular weight cutoff of 5,000 KD (Fig. 2). The inflow and outflow lines were constructed of hollow 170μm OD silica tubing (SGE, Austin, TX) glued into the dialysis fiber.

Norepinephrine (1nmol/μl) was diluted in normal saline with 0.1% sodium ascorbate. Norepinephrine was then perfused into the microdialysis probe at a rate of 5μl/min with a microinfusion pump (BAS) to confirm functionally the position of the probe in the SA node. When properly positioned, the heart rate routinely increased by 30-40 bpm within 30 seconds of introducing the norepinephrine. This tachycardia was not observed when the probe was not optimally placed in the SA node. The norepinephrine was immediately flushed out, and the system was perfused with saline (5μl/min) for 1 hour and permitted to re-equilibrate before beginning the

experimental protocol. Previous studies have demonstrated that one hour is needed post insertion of the probe to allow restoration of baseline interstitial conditions (Van Wylen et al., 1990). As further validation of the probe location, the probe was removed and repositioned parallel to but 1-2mm distant from the original insertion at the conclusion of nine experiments. No significant changes in heart rate were observed at the new probe position, when norepinephrine was reintroduced. In addition, preliminary unpublished micrography of the SA node tissue around the probe is consistent with the expected nodal histological organization (Masson-Pevet et al., 1984; Opthof et al., 1987).

Protocol I: Increasing Endogenous MEAP Release

In previous studies, MEAP was able to inhibit vagal function when infused by microdialysis into the SA node (Farias et al., 2000). This inhibition involved opiate receptors since it was blocked by the high affinity opiate antagonist, diprenorphine (Farias et al., 2000). Since added MEAP modulates vagal function during local perfusion of the SA node, can MEAP be identified as a normal constituent of the SA node; can its content be altered locally. A schematic diagram of the protocol is illustrated (Fig. 3). A suture was placed around the sinoatrial node artery to allow for its reversible occlusion (Fig. 1). A 10-min initial dialysate collection was made. Leucine-arginine (leu-arg: 30nmol/μl) was then added to the microdialysis perfusate to reduce the enzymatic breakdown of MEAP and improve the recovery of endogenous peptides in the dialysate. After 5-min preinfusion, a 10-min control collection was made and then the sinoatrial node artery was temporarily occluded to

produce four 10-min periods of reduced blood flow each followed by a 10-min reperfusion. A 30-min prolonged occlusion and 30-min reperfusion period followed the "preconditioning-like" sequences. During each 10-min interval, the dialysate was collected and the MEAP content was determined by radioimmunoassay.

Protocol II: Alteration in Vagal Function

Vagal function was evaluated prior to beginning the protocol and at selected intervals thereafter as indicated by the vertical arrows in Fig. 3. The vagus was stimulated at a supra-maximal voltage (15v) for 15 seconds at 3 Hz. The vagus was evaluated before and after initiating the leu-arg infusion. The vagus was also evaluated after the "preconditioning-like" protocol, during the 30-min prolonged occlusion (at 15-min), and during the 30-min reperfusion period (at 15-min). After each vagal stimulation, the vagal changes in heart rate were recorded online.

Protocol III: Opiate Receptor Involvement

Previous research has indicated that opioids are involved in the myocardial protection produced, during preconditioning (Liang et al., 1999; Schultz et al., 1998). A delta opioid receptor has been implicated as responsible for some of the beneficial effects of preconditioning in rat ventricle (Schultz et al., 1997a; Schultz et al., 1997b). The protocol described above was modified (Fig. 4) to add a second 30-min occlusion. During the second occlusion period the vagus nerve was re-evaluated at 15-min as previously described and again at 20, 25 and 30 minutes. After the first stimulation at 15-min, naltrindole (0.22mM), a selective delta opioid receptor antagonist was infused via the dialysis probe and the vagus was re-evaluated again at

20-min. In half of the animals protocol IV was conducted first and naltrindole was initiated afterward at 20-min and the vagus was re-evaluated at 25-min. The responses to naltrindole were identical regardless of the protocol order and results from the two groups were combined. The dose of naltrindole had been previously determined to block the vagolytic effect of exogenous MEAP.

Protocol IV: K_{ATP} Channel Involvement

Preconditioning effects are elicited in part through an apparent opioid activation of ATP sensitive potassium (K_{ATP}) channels (Gross et al., 1992). Blockade of the K_{ATP} channels has been shown by Gross et al (1992) to prevent myocardial preconditioning in rats. Opiates have also been shown to be capable of activating K_{ATP} channels within the heart and this activation was reversed by infusion of the selective delta receptor antagonist, naltrindole.

The protocol described in Fig. 4 was employed again except that once, the vagal responses were recorded after 15-min of the final occlusion, a selective K_{ATP} channel blocker, glibenclamide (0.22mM) was substituted for the naltrindole described above. After this period, the vagus was re-evaluated at 20-min. In half of the animals studied, protocol III (see above) was conducted first and glibenclamide was initiated afterwards at 20-min and the vagus was re-evaluated at 25-min. The responses to glibenclamide were identical regardless of the protocol order and results from the two groups were combined. In protocols III and IV glibenclamide and naltrindole were combined and infused into the dialysis probe 5-min prior to a fourth stimulation conducted at 30-min.

MEASUREMENT OF MEAP:

MEAP in the dialysate was measured by radioimmunoassay (Younes et al., 2000). This assay employs an antiserum obtained from rabbits immunized with a synthetic peptide corresponding to the C-terminal 15 amino acid sequence of proenkephalin. This sequence includes the C-terminal heptapeptide MEAP. The peptide was conjugated to keyhole limpet hemocyanin. The resulting antiserum specifically recognizes the C-terminus of MEAP with less than 1% crossreactivity with all closely related low molecular weight enkephalins likely to be present in the dialysate. The assay does not distinguish between MEAP and its immediate metabolite des-tyrosyl-MEAP. The immediate metabolite des-tyrosyl-MEAP is not biologically active. Synthetic MEAP was radioiodinated by chloramine-T and a double antibody radioimmunoassay was conducted. Bound and free ligands were separated by immune-precipitation with anti-rabbit gamma globulin (second antibody).

MATERIALS:

MEAP was synthesized by American Peptide Co, in Sunnyvale, CA; naltrindole and glibenclamide were obtained from Sigma RBI in St. Louis, MO. All microdialysis probes were fabricated by Dr. David G. L. Van Wylen of St. Olaf College in St. Olaf, Minnesota (Van Wylen et al., 1990).

DATA ANALYSIS:

The data were expressed as mean ± standard error. Differences were evaluated by analysis of variance and multiple post-hoc comparisons were made with Tukey's

test (GB-STAT™, Dynamic Microsystems, Silver Spring, MD). Repeated measures designs were used where appropriate. $P < 0.05$ was accepted as a statistically significant difference.

RESULTS:

Twenty animals were studied; of these, sixteen animals completed the experimental protocols. The remaining four animals were excluded from the final analysis, in the absence of acceptable baseline vagal responses. There were no statiscally significant differences in starting heart rate (HR) and mean arterial pressure (MAP) during occlusion (138±2 & 98±3 HR & MAP), and reperfusion (135±3 & 100±2 HR & MAP) as compared to control (132±4 & 97±3 HR & MAP). Initial MEAP measurements prior to occlusion were consistently very low; near the detection limits of the assay. There was a statistically significant increase in MEAP concentration, during each of the four occlusion periods (Fig. 5) when compared to baseline. However, in each case the values returned to near control, during each subsequent reperfusion. There were no differences between MEAP concentration during any of the 10-min reperfusion periods and the original control values (Fig. 5). There was an increase in the recovered MEAP as the preconditioning-like protocol proceeded through the four alternating bouts of occlusion and reperfusion. There were no statiscally significant differences observed when the responses to right vagal stimulation were compared during control saline infusion (-27bpm ± 3.0:C), during leu-arg infusion (-21bpm ± 3.0: CLA) into the SA node, and after completing the four-cycle preconditioning-like protocol (-32bpm ± 1.7:PC). However, there was a

statiscally significant improvement or augmentation in vagal bradycardia, during the prolonged 30-min occlusion (-56bpm ± 2.4:15mO), as compared to control (Fig. 6). The vagally induced bradycardia returned to control values (-35bpm ± 1.8:15mR) during the subsequent 30-min reperfusion.

Delta Receptor Involvement

The vagal response data is summarized in Figure 7. In the first subset of subjects (n = 8), a second 30-min occlusion was conducted and the enhanced vagal response was observed again after 15-min of occlusion (-57bpm ± 2.3:15mO). The delta opiate receptor antagonist, naltrindole (0.22mM) was then introduced into the nodal perfusate. After an additional, 5-min of occlusion, the vagal response was retested. The enhanced vagal response observed during occlusion was completely reversed by nodal infusion of naltrindole (-19bpm ± 1.8:NT).

K_{ATP} Channel Involvement

In this subset of subjects (n = 8), the K_{ATP} channel antagonist, glibenclamide (0.22mM), was substituted for naltrindole as described in the prior experiment. Infusion of glibenclamide into the sinoatrial node partially blocked the augmentation of vagally mediated bradycardia (Fig. 7). There was a return towards control values in the presence of glibenclamide, however there was not a complete reversal of the enhanced vagal response (-43 ± 1.0:Glib vs. –27bpm ± 2.0:C). Given that the reversal with glibenclamide was only partial, the activation of the K_{ATP} channels may be only one of several contributors to the response. Although the concentration of glibenclamide was substantial, there was no practical way to evaluate the

completeness of the resulting K_{ATP} channel blockade. When naltrindole and glibenclamide were combined prior to a third, vagal test at 25-min of occlusion, the two effects were not additive and the combination was not different from naltrindole alone (–20 ± 2.0: NT + Glib: n = 4).

DISCUSSION:

The initial goal of this study was to determine if MEAP could be identified locally in the SA node, and could the local release of MEAP be modified. This question was formulated from earlier reports that MEAP was abundant in the myocardium (Barron et al., 1992) and was highly vagolytic when administered systemically or directly into the SA node (Caffrey et al., 1999, Farias et al., 2001). In the current study, MEAP was recovered in the nodal dialysate. The concentration of MEAP released was very low but rose dramatically in the dialysate during each occlusion of the SA node artery. The mass of enkephalin released also appears to increase gradually during sequential occlusions suggesting an increasing capacity for synthesis and/or release. An increased capacity for enkephalin release during repeated ischemic episodes might be consistent with the putative role of opioids in ischemic preconditioning.

The findings obtained in the second part of the study represent something of a departure from the expected results. The original hypothesis suggested that an interruption or reduction in vagal control should accompany a local nodal increase in endogenous MEAP release. The hypothesis was based on the previous observation that exogenous MEAP delivered by dialysis to the SA node suppressed vagal control (Farias et al., 2001). However, when the endogenous MEAP was elevated during occlusion of the SA node artery, no vagal impairment was observed. In contrast, vagal function was consistently improved instead. These apparently contradictory observations suggest that the increase in endogenous peptide is either coincident and

unrelated to the vagal improvement or that the system or circumstances are sufficiently complex that the endogenous and exogenous peptides produce very different responses, during normoxic and ischemic conditions.

The enhanced vagal response during occlusion does appear to involve opioid receptors and would be consistent with the observations made by others during preconditioning (Liang et al., 1999; Schultz et al., 1998) in the ventricle. Ischemic preconditioning is a phenomenon in which brief periods of ischemia and reperfusion protect the myocardium against cell injury caused by subsequent prolonged ischemia (Dana et al., 1988). Opioids have been linked to the beneficial effects produced by ischemic preconditioning (Schultz et al., 1998). The opioid contribution to preconditioning was linked to delta opioid receptors in rats, since naltrindole was able to block cardioprotection in ischemicly preconditioned rats (Schultz et al., 1997a; Schultz et al., 1997b). In the present study there was a complete restoration of the control vagal response when the selective delta opioid receptor antagonist, naltrindole, was infused into the SA node. This reversal of the augmented vagal response by naltrindole suggests that the delta opioid receptor is involved during SA nodal ischemia as well.

The similarities between the observations made here and those made by others during ischemic preconditioning suggested that the two processes might be related. Since the ATP sensitive potassium (K_{ATP}) channels have been implicated in ischemic preconditioning (Gross et al., 1992; Liang et al., 1999), these channels might also be involved in the improved vagal response reported here. In addition to opioids, there

are several other known activators of the K_{ATP} channel, including; mild acidosis, acetylcholine, adenosine, and several other g-protein coupled processes (Hiraoka, 1997). The current study has demonstrated an increase in the cardiac opioid, MEAP, in the nodal interstitium during occlusion of the nodal artery. This local hypoperfusion was also accompanied by an augmentation of vagal function, when compared to control. The improved vagal effect may involve the activation of a K_{ATP} channel, since glibenclamide (a selective K_{ATP} channel antagonist) partially reversed the augmented vagal bradycardia during local nodal increases in endogenous MEAP. Intracellular ATP concentrations and perhaps more specifically a high ATP/ADP ratio normally inhibit the K_{ATP} channel (Hiraoka, 1997). During ischemia the ATP/ADP ratio decreases, presumably reducing the inhibitory effects of intracellular ATP on the K_{ATP} channel (Hiraoka, 1997). Opioids have been suggested as being capable of activating this channel through coupling with opioid receptors, perhaps making the channel more responsive to the decline in ATP during ischemia (Hiraoka, 1997; Gross et al., 1992).

Exogenous infusion of MEAP inhibits vagally mediated bradycardia locally when administered by dialysis into the SA node (Farias et al., 2001). In contrast, increases in endogenous MEAP produced by ischemia seem to augment vagal control of heart rate. One conclusion that may explain these apparently disparate observations would be that the increased MEAP and the increased vagal function were coincident but unrelated. However, several observations argue against that interpretation. First, the augmentation is temporally observed only when the MEAP is elevated. Second,

the degree of augmentation is quantitatively reversed by the opiate antagonist, naltrindole. Although another opioid could be responsible for the improved response to vagal stimulation, the same delta selective antagonist blocks both the vagolytic and vagal enhancing effects. This suggests that that different targets (e.g. nerves vs nodal cells) and/or local conditions (normoxia vs ischemia) may be contributory.

The opposite response to endogenous and exogenous MEAP might be consistent if the presumed opiate receptors were localized on different cell types. Each cell type might have responded to the opioids in different dose ranges. This however, seems unlikely since careful dose responses to exogenous MEAP conducted in prior studies, provided no indication of an enhanced vagal effect at any applied dosage. If the competing receptors are located on different cell types, perhaps the inhibitory opiate receptors on prejunctional nerve endings inactivate or down regulate during ischemia. They might then eliminate the vagolytic effect and in the process expose the observed facilitory effect mediated by post-junctional opiate receptors on the pacemaker cells. Alternatively, ischemia may relocate the primary nodal pacemaker to a group of nodal cells either more sensitive to the vagus and/or more distant from the influence of the locally generated MEAP. Sorting out these and a number of other more speculative explanations will necessarily require a number of additional studies.

If the opiates are important regulators of normal vagal activity, then opioid dysfunction may be an important contributor to cardiovascular disease. A number of cardiovascular disease processes are associated with an imbalance in autonomic

function (Binkley et al., 1991). Although abnormal sympathetic activity is often identified as a major contributor to the disease process, as in heart failure, these diseases are usually also accompanied by abnormal vagal function (Binkley et al., 1993). Following myocardial infarction, those who quickly regain vagal control of the heart have a much better long-term prognosis (Kleiger et al., 1987). As such, the nodal opioids may provide new insights into the nodal environment and the regulation of the cardiac rhythm. This work may also assist in the identification of new strategies and therapeutic approaches to improve survival in the post-ischemic heart.

In conclusion, there was a repeated increase in the concentration of the cardiac opioid, MEAP, during repeated occlusion, which returns to baseline during reperfusion. The increase in nodal MEAP during nodal hypoperfusion was accompanied by an augmentation of vagal function. Vagal function was only enhanced when MEAP concentrations were increased. The delta opioid receptor antagonist, naltrindole, completely abolished the augmented vagal response, while the augmentation in vagal function was partially reversed by the K_{ATP} channel antagonist, glibenclamide. Therefore, the data then supports the hypothesis that local nodal enkephalins increase during nodal hypoperfusion and improve vagal control of heart rate through an interaction with delta opiate receptors mediated in part by the activation of K_{ATP} channels.

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LEGEND

Fig. 1. This figure provides a diagrammatic representation of the sinoatrial nodal area, the microdialysis probe and the site of arterial occlusion.

Fig. 2. This figure provides a diagrammatic illustration of the microdialysis probe and its dimensions.

Fig.3. This figure provides a schematic diagram of the preconditioning-like protocol employed in protocols I and II. The arrows indicate times when the vagus nerve was stimulated.

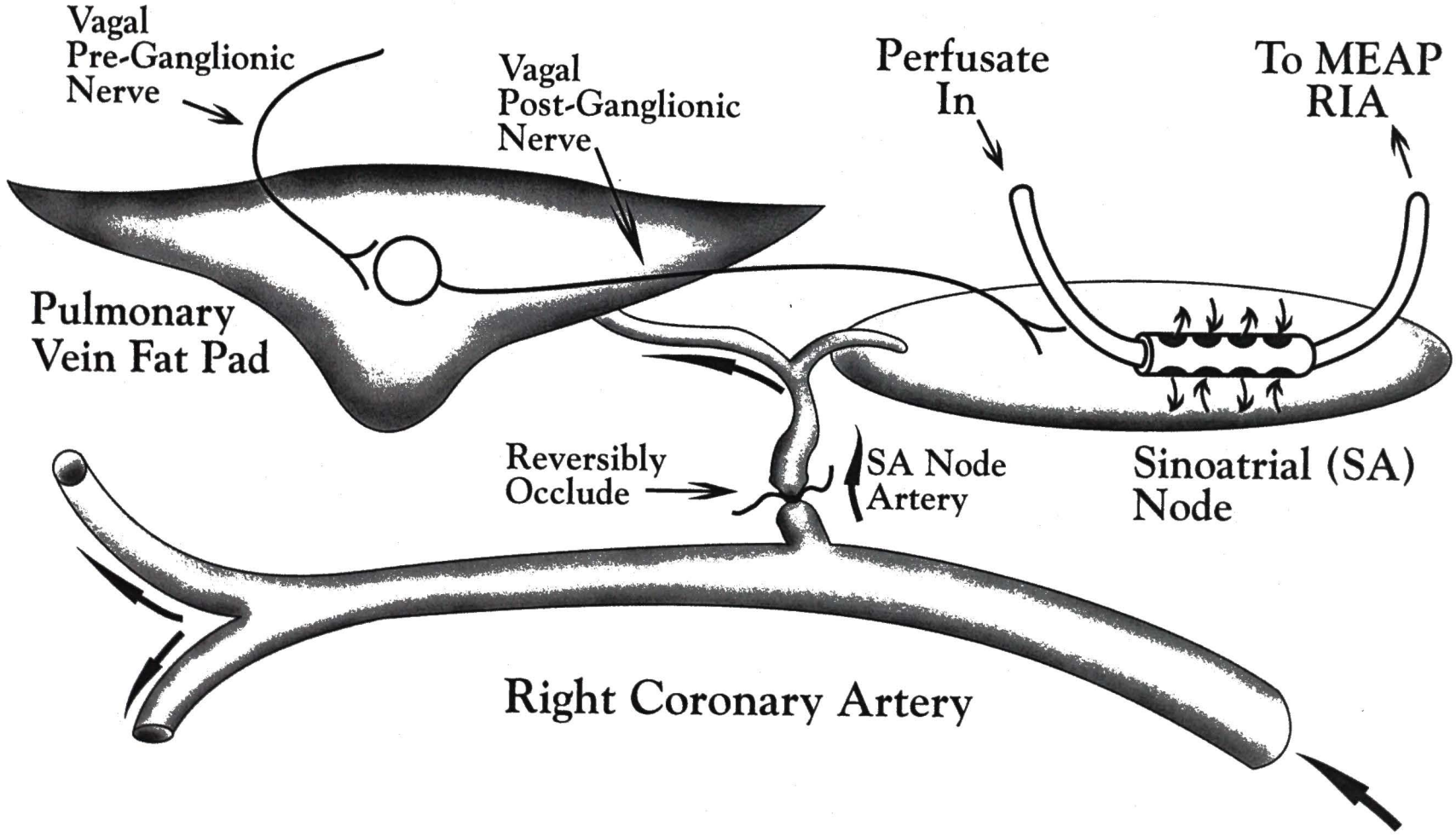
Fig. 4. This figure is a diagram of the preconditioning-like protocol as modified for protocols III and IV. Arrows indicate times when the vagus nerve was stimulated. A second 30-min SA node artery occlusion was added to the original experimental protocol and the vagus nerve was re-evaluated at 15, 20, 25, and 30 minutes during the added occlusion. A nodal infusion of naltrindole or glibenclamide was begun at 15 or 20 minutes. The two drugs were combined at 25-min.

Fig. 5. This figure illustrates the local nodal MEAP recovered in the dialysate during control saline perfusion (CS), during leucine-arginine (LA) vehicle perfusion of the node, during four 10-min occlusions (O1, O2, O3, O4) intermixed with four 10-min reperfusions (R1, R2, R3, R4), and during a prolonged 30-min occlusion period (10mO, 20mO, 30mO) followed by a 30-min reperfusion period (10mR, 20mR, 30mR). There was a repeated increase in nodal MEAP release during the preconditioning-like protocol that returned to low control values during reperfusion. There was a sustained increase in MEAP throughout the 30-min occlusion, which

also returned to baseline during the 30-min reperfusion period. Data were expressed as mean ± SE and the arrows indicate times when the vagus nerve was stimulated. The (*) indicates statistically significant differences from control (n = 16).

Fig. 6. This figure illustrates the vagally mediated changes in heart rate produced during right vagal stimulation at selected points in protocols I and II. Vagal function was augmented during the 30-min prolonged occlusion, which correlated with elevated MEAP in the nodal dialysate. There was a return towards control vagal response, during the 30-min reperfusion, when the local nodal MEAP had decreased to control (values are means ± SE, n=16). The (*) indicates significant differences from control ($P<0.05$).

Fig. 7. This figure illustrates the decrease in heart rate during right vagal stimulation at selected points in protocols III-IV. Vagal function was again augmented during local reductions in nodal blood flow. This augmentation was totally reversed by a 0.22mM naltrindole (NT: n = 8) infusion into the probe and partially reversed by a 0.22mM glibenclamide (Glib: n = 8) infusion. When combined (NT + Glib: n = 4) the vagal response was not additive and was identical to NT alone. Data were expressed as mean ± SE where (*) indicates statistically significant differences from the control and (#) indicates statistically significant differences from the second 15-min occlusion (15mO).


Vagal
Pre-Ganglionic
Nerve
Vagal
Post-Ganglionic
Nerve
Perfusate
In
To MEAP
RIA
Pulmonary
Vein Fat Pad
Reversibly
Occlude
SA Node
Artery
Sinoatrial (SA)
Node
Right Coronary Artery

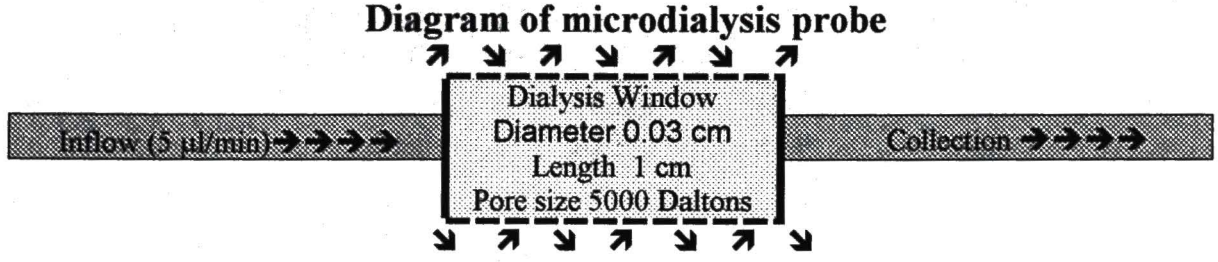
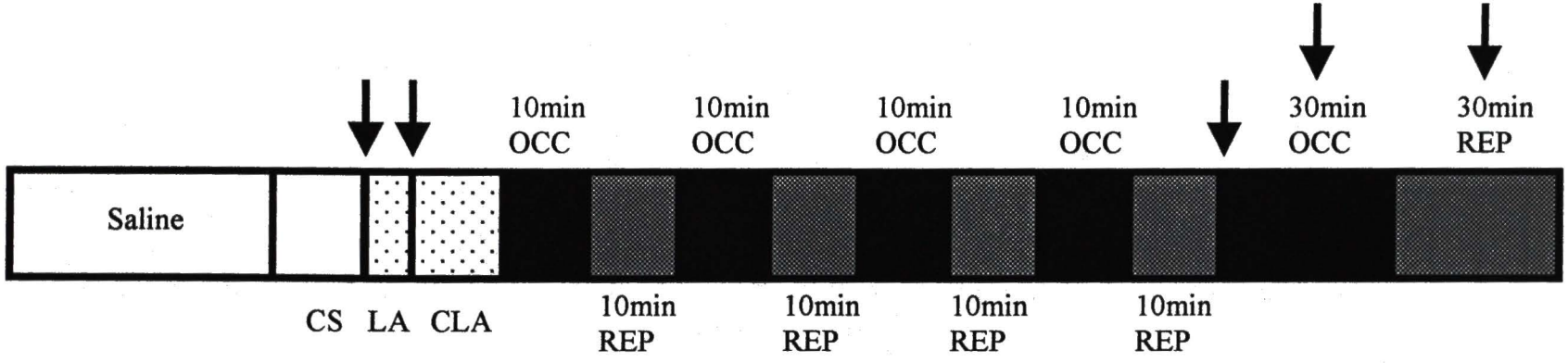


Diagram of microdialysis probe
Dialysis Window
Diameter 0.03 cm
Length 1 cm
Pore size 5000 Daltons
Inflow (5 µl/min)
Collection

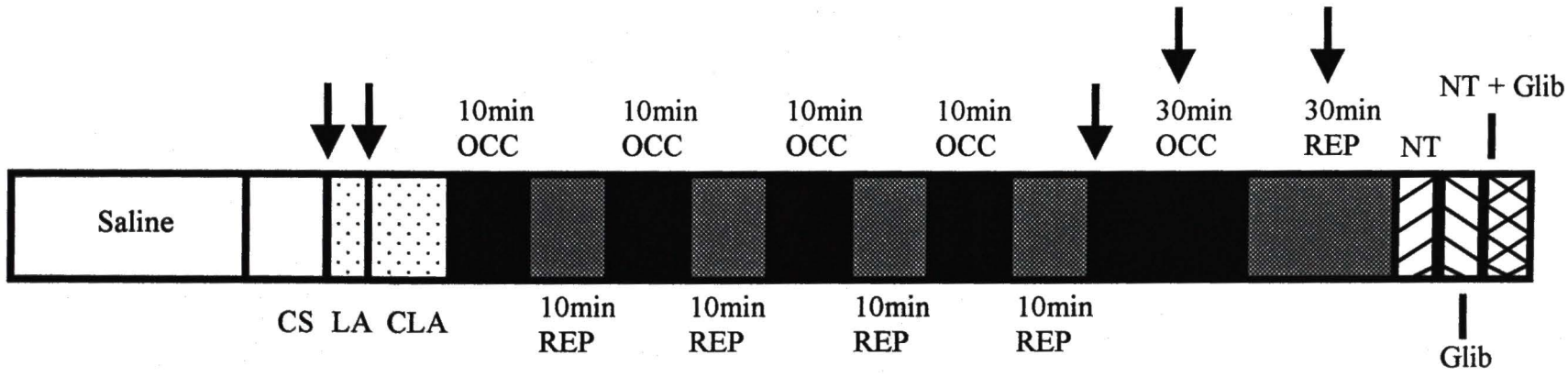
Figure 2



Legend:

- 1 Hour Saline Infusion (Saline)
- 10 minute control collection with saline (CS)
- 5 min Leu-arg infusion (LA)
- 10 min control collection with leu-arg (CLA)
- 10 minute occlusion (10min OCC)
- 10 minute reperfusion (10min OCC)
- 30 minute occlusion (30min OCC)
- 30 minute reperfusion (30min REP)
- Vagal Stimulation

Figure 3



Legend:

- 1 Hour Saline Infusion (Saline)
- 10 minute control collection with saline (CS)
- 5 min Leu-arg infusion (LA)
- 10 min control collection with leu-arg (CLA)
- 10 minute occlusion (10min OCC)
- 10 minute reperfusion (10min OCC)
- 30 minute occlusion (30min OCC)
- 30 minute reperfusion (30min REP)
- Vagal Stimulation
- 5 minute Naltrindole Infusion (NT)
- 5 minute Glibenclamide Infusion (Glib)
- 5 minute NT + Glib Infusion (NT + Glib)

Figure 4

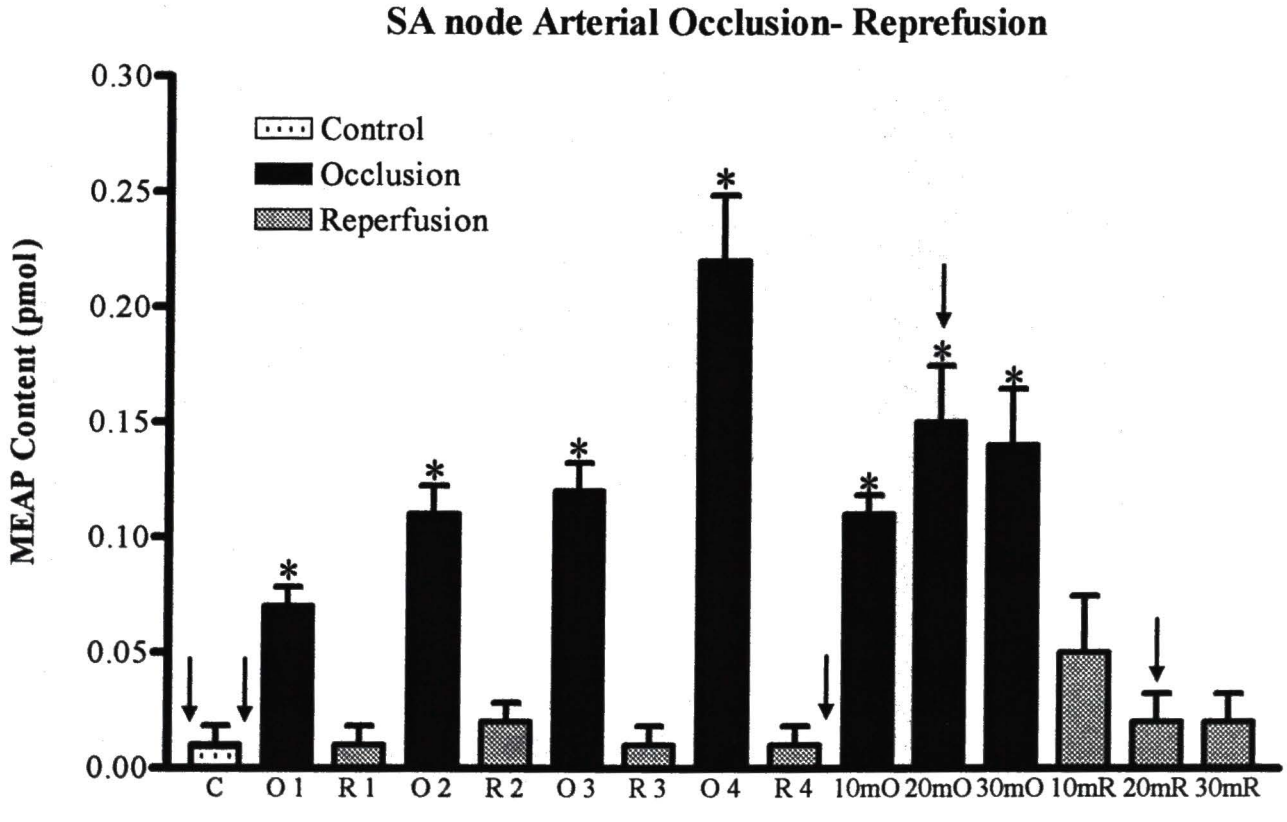

SA node Arterial Occlusion- Reprefusion
Control
Occlusion
Reperfusion
MEAP Content (pmol)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
C
O 1
R 1
O 2
R 2
O 3
R 3
O 4
R 4
10mO
20mO
30mO
10mR
20mR
30mR
Time (min)

Figure 5

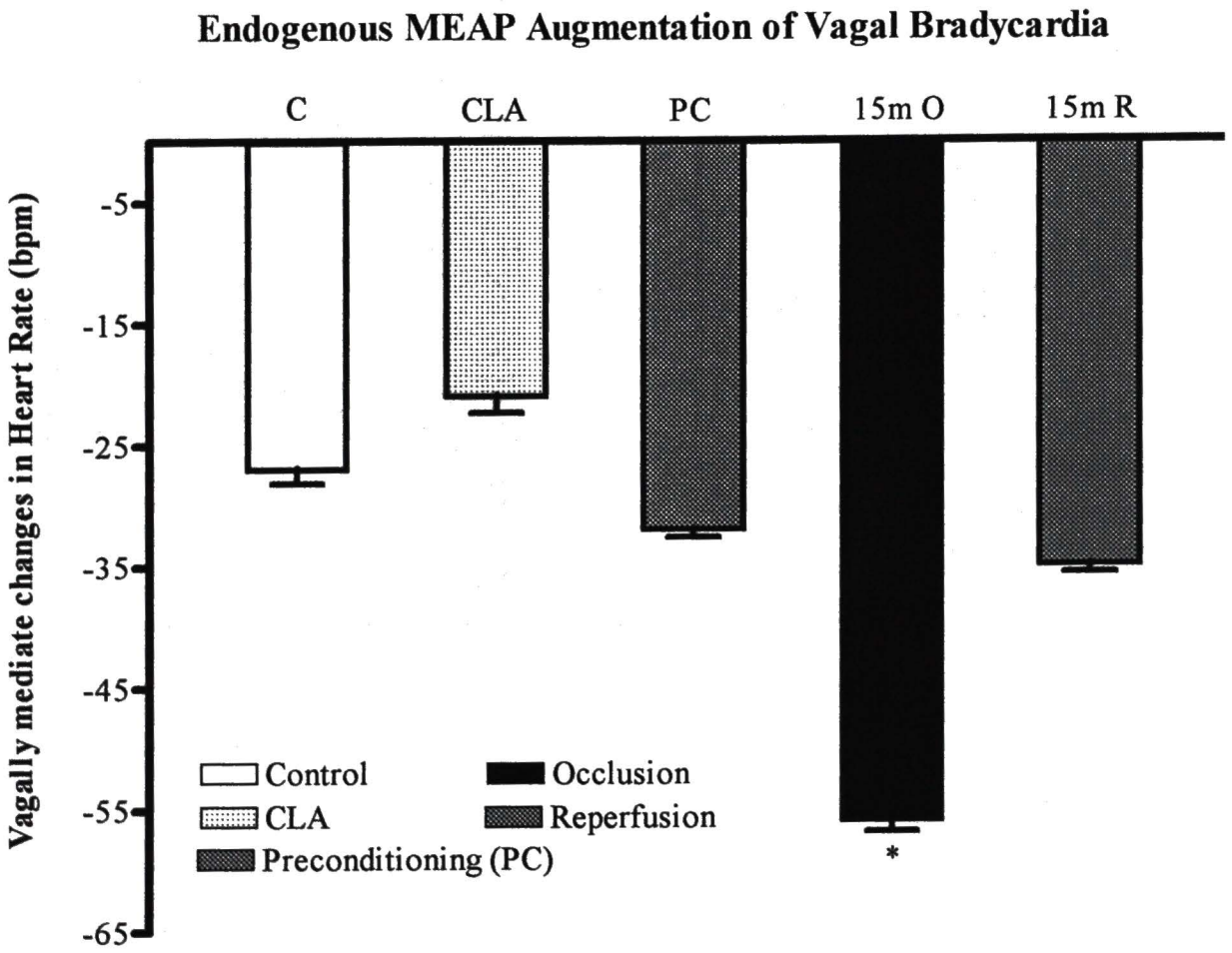

Endogenous MEAP Augmentation of Vagal Bradycardia
C
CLA
PC
15m O
15m R
Vagally mediate changes in Heart Rate (bpm)
-5
-15
-25
-35
-45
-55
-65
Control
Occlusion
CLA
Reperfusion
Preconditioning (PC)
*

Figure 6

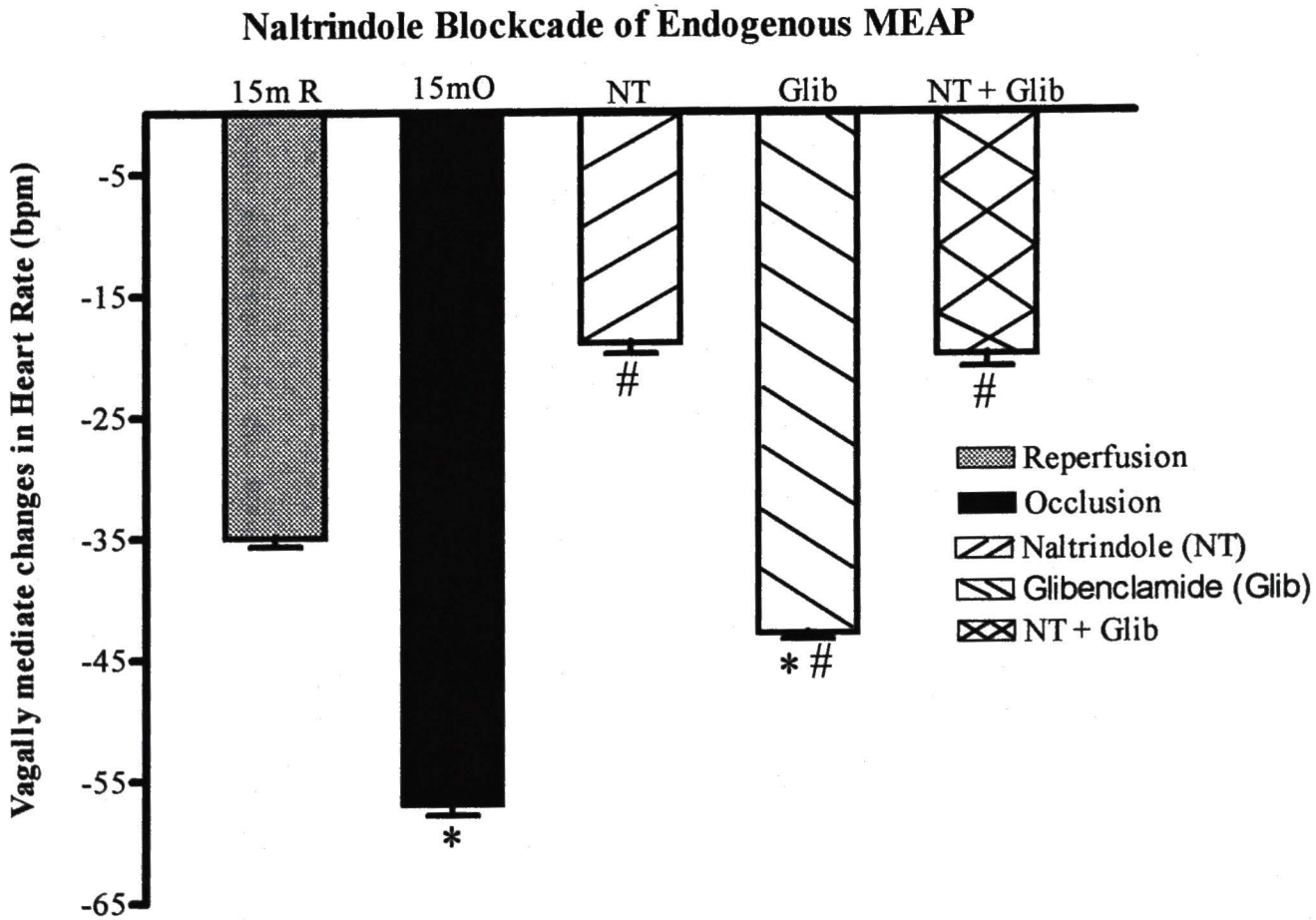

Naltrindole Blockcade of Endogenous MEAP
15m R
15mO
NT
Glib
NT + Glib
Vagally mediate changes in Heart Rate (bpm)
-5
-15
-25
-35
-45
-55
-65
*
#
#
*#
Reperfusion
Occlusion
Naltrindole (NT)
Glibenclamide (Glib)
NT + Glib

Figure 7

CHAPTER IV

We began with two basic questions: 1) which opiate receptor was responsible for the vagolytic effect of MEAP and 2) can endogenous MEAP be shown to produce a similar vagolytic effect. The major findings of this work were as follows:

1) There was a dose dependent inhibition of vagal bradycardia during MEAP infusion into the SA node. The maximum vagolytic effects of exogenous MEAP were elicited at a dose of 0.3mM.
2) There was a dose dependent inhibition of the vagolytic effects of MEAP during the coinfusion of the selective delta opioid receptor antagonist, naltrindole. The maximum reversal of MEAP was elicited at a dose of 0.07mM.
3) The vagolytic effect of MEAP was replicated by the selective delta opioid receptor agonist, deltorphin, and deltorphin was more efficacious than MEAP.
4) Deltorphin's vagolytic effect was reversed by doses of naltrindole similar to those employed in the coinfusion experiments of MEAP and naltrindole.
5) Other opioid agonist and antagonist were ineffective at altering the control vagal responses or the vagal inhibition by MEAP.
6) There was an increase in endogenous MEAP recovered in the dialysate during reduced nodal blood flow. This increase returned towards control concentrations, during reperfusion.

7) There was an enhanced vagal bradycardia, during occlusion of the sinoatrial node artery. This enhanced vagal response was returned towards control, during vascular reperfusion of the sinoatrial node.

8) The enhanced vagal responses during reduced blood flow were restored to control with a local 0.07mM infusion of naltrindole into the sinoatrial node.

9) There was a partial return of the enhanced vagal response towards control, during local infusion of the K_{ATP} channel blocker, glibenclamide.

10) There were no statiscally significant differences between the paired infusion of glibenclamide and naltrindole and naltrindole alone.

The inhibition of the vagus by exogenous MEAP was very short-lived. There was a return towards control vagal responses within two minutes post washout. This selective vagal inhibition appears to be elicited through a delta opioid receptor mechanism. What is very interesting was that despite similar dose effects, time courses and reversal by naltrindole, the more selective delta agonist, deltorphin was more efficacious, when compared to MEAP.

The enhanced vagal bradycardia during nodal artery occlusion could be beneficial since it occurs during a reduced supply of fuel. The vagolytic effect could contribute further to myocardial protection by opposing the enhanced sympathetic outflow during ischemia. The heart would be protected from the increased energy costs associated with enhanced sympathetic activity.

CHAPTER V

PROPOSAL OF FURTHER RESEARCH

The following studies are proposed to further clarify the cardiovascular effects of the enkephalin, MEAP:

1) Infuse selective delta 1 and 2 receptor agonist and antagonist to determine whether the inhibition of vagal bradycardia by MEAP is a delta 1 or a delta 2 selective mechanism.
2) Determine the transfer rate of the microdialysis probe in order to determine the concentration of MEAP, selective opioid agonists and selective antagonists in the sinoatrial node. This data will also help to estimate the nodal concentrations of the MEAP measured, during hypoperfusion.
3) Infuse radioactive peptide into the node to determine the area of influence of the probe and to quantify the number of receptors present in the node during normoxia and low flow.
4) Infuse other selective agonists and antagonists into the probe during low flow, to determine the possible contributions of the other opioid receptors.
5) Infuse various doses of glibenclamide into the probe during low flow, to determine its complete dose effect relationship. The full dose response is needed to determine if the partial effect of glibenclamide was the result of an inadequate dose.

6) Infuse exogenous MEAP into the probe during low flow, to determine if exogenous MEAP is effective (vagolytic) during low flow conditions.

7) Chromatograph the MEAP recovered from the node to verify it's molecular form and biological activity.

8) Utilize fluorescent labeled deltorphin to identify the nodal cell types which express the opiate receptors responsible for the observed biological effects.

APPENDIX

We described a paradoxical effect of MEAP in the two studies presented. In the delta receptor studies, infusion of exogenous MEAP into the sinoatrial node was observed to be vagolytic (Figure 1:p.47, Figure 2:p.48). However, an increase in endogenous MEAP (Figure 5:p.88) appears to augment vagal function (Figure 6:p.89). There are a number of possible mechanisms that could mediate the observed, functionally different effects of MEAP. Some of the mechanisms are more speculative than others. As a point of clarity, we have illustrated three possible mechanisms, which could explain these disparate MEAP effects. First the delta receptors on the vagal nerve terminals and on the sinoatrial node cells might be coupled differentially (108). If the delta receptors on the nerve terminals were coupled to a potassium and/or calcium channel, the infusion of exogenous MEAP could produce hyperpolorization of the nerve terminal and/or a decrease in vesicular release of acetylcholine. Overall, this would produce an inhibition of the vagal transmission during normoxic conditions. If the receptor on the sinoatrial node were differentially coupled to K_{ATP} channels, they would be difficult to open during normoxia when ATP is abundant. During conditions such as ischemia, when the ATP/ADP ratio is decreased, the nodal cell K_{ATP} channel may be more responsive to activation by opioids. Hyperpolorization of the sinoatrial node might then enhance the response to vagal stimulation. Second, there could be a difference in receptor population during reduced blood flow vs normoxia (109, 110). During normoxia,

there may be more available receptors on the nerve terminal compared to the sinoatrial node cells (109). For instance, if the nodal cell receptors were sequestered in a subcellular compartment, then they would be unavailable for stimulation. Added MEAP would then inhibit vagal bradycardia by reducing acetylcholine release as described above. During hypoxia, an upregulation of nodal cell receptors (110) might then expose a second site of action. The nodal cell receptors might then facilitate the effect of acetylcholine by hyperpolarizing the nodal cells. Therefore, the acetylcholine that was released would be more effective. Third, it is possible that the increase in endogenous MEAP and augmented vagal function are coincident, but unrelated (111). An unidentified opioid might mediate the augmented vagal response during hypoxia, and mask the vagolytic effect of endogenous MEAP? However, without additional studies the vagal facilitation and the precise target for vagolytic effect remain undefined.

Table 1: Control Hemodynamic Measurements of the Receptor Profile Studies

Dog #	Control		Vagal Stimulation		Baseline (Probe Inserted)		Vagal Stimulation	
	HR	MAP	ΔHR	MAP	HR	MAP	ΔHR	MAP
3787	120	92	59 (119-60)	90	118	102	57 (117-60)	101
3816	133	110	42 (132-90)	109	130	108	40 (129-89)	109
3815	145	125	58 (143-85)	122	143	122	60 (140-80)	121
3781	142	120	50 (140-90)	119	139	120	48 (138-90)	117
3818	138	117	46 (138-92)	115	137	119	43 (138-95)	115
3822	139	121	69 (139-70)	119	136	125	67 (138-71)	123
3832	129	101	65 (128-63)	102	131	103	70 (130-60)	100
3871	136	85	37 (136-99)	88	135	90	37 (134-97)	92
3892	150	91	30 (150-120)	92	151	89	20 (151-131)	88
3889	120	92	42 (119-77)	95	110	90	44 (110-66)	94
3897	132	70	30 (132-102)	73	133	72	23 (133-110)	74
3976	115	62	57 (114-57)	60	111	61	55 (111-56)	60
3974	150	99	59 (150-91)	98	145	103	57 (144-87)	102
4029	133	93	53 (130-77)	95	129	98	58 (129-71)	92
4031	145	113	81 (140-59)	110	142	111	77 (142-65)	112
4045	130	88	30 (130-100)	89	136	85	29 (136-107)	84

There were no statiscally significant changes in heart rate (HR), mean arterial Pressure (BP), and control vagal stimulation, during the receptor profile studies.

Table 1 (continued): Control Hemodynamic Measurements of the Receptor Profile Studies

Dog #	Control		Vagal Stimulation		Baseline (Probe Inserted)		Vagal Stimulation	
	HR	MAP	ΔHR	MAP	HR	MAP	ΔHR	MAP
4043	160	92	54 (160-106)	95	163	92	52 (163-111)	95
4051	147	91	32 (144-112)	92	145	93	33 (144-111)	97
4055	128	64	63 (126-63)	65	125	61	62 (125-63)	62
4071	135	81	24 (134-110)	82	134	82	35 (135-100)	85
4063	139	110	37 (140-103)	109	137	103	36 (138-102)	105
4079	149	86	69 (149-80)	85	143	90	70 (143-73)	92
4064	140	116	19 (133-114)	120	135	112	18 (134-116)	115
4086	136	59	52 (132-80)	63	132	70	40 (130-90)	65
4082	140	97	50 (140-90)	98	145	101	40 (148-108)	102
4095	135	112	23 (131-108)	110	131	109	30 (131-101)	102
4094	144	81	49 (144-95)	82	140	108	32 (140-108)	105
4100	135	84	29 (130-101)	88	130	104	30 (130-100)	90
4096	159	120	37 (160-123)	118	157	115	37 (155-118)	112

There were no statiscally significant changes in heart rate (HR), mean arterial pressure (MAP), and control vagal stimulation during the receptor profile studies.

Table 1 (continued): Control Hemodynamic Measurements of the Receptor Profile Studies

Dog #	Control		Vagal Stimulation		Baseline (Probe Inserted)		Vagal Stimulation	
	HR	MAP	ΔHR	MAP	HR	MAP	ΔHR	MAP
4112	120	50	50 (120-70)	55	112	62	42 (112-70)	63
4103	127	80	20 (130-110)	83	115	93	18 (114-96)	92
4109	118	60	24 (110-86)	62	108	81	54 (108-54)	65
4122	131	103	30 (130-70)	101	125	76	25 (125-100)	75
4117	121	52	50 (121-71)	55	119	60	53 (119-66)	62
4120	140	96	40 (140-100)	91	138	90	37 (132-96)	93
4126	140	112	60 (140-80)	110	139	106	59 (139-80)	105
4124	142	120	28 (138-112)	119	140	122	31 (139-108)	119
4137	141	79	34 (140-106)	77	143	72	38 (145-107)	68

There were no statiscally significant changes in heart rate (HR), mean arterial pressure (MAP), and control vagal stimulation during the receptor profile studies (n = 37).

Table 2: Average Heart Rate and Blood Pressure Measurements of the Receptor Profile Studies

	Control		Vagal Stimulation		Baseline (Probe Inserted)		Vagal Stimulation	
	HR	MAP	ΔHR	MAP	HR	BP	ΔHR	MAP
MEAN	136	93	44	93	134	95	44	94
SD	11	21	16	19	12	19	16	18
SE	0.3	0.6	0.4	0.5	0.3	0.5	0.4	0.5

This table represents the mean, standard deviation (SD), and standard error (SE) from the data described in table 1.

Table 3: Average Measurements from Delta Receptor Studies

Nodal Perfusate			Baseline After Washout (with saline)	
	HR ± SE	MAP ± SE	HR ± SE	MAP ± SE
Saline	139 ± 0.3	97 ± 0.5		
0.01mM MEAP	140 ± 2	102 ± 1	138 ± 1	101 ± 2
0.03mM MEAP	138 ± 2	103 ± 1	137 ± 2	102 ± 1
0.1mM MEAP	136 ± 2	105 ± 1	135 ± 3	100 ± 0.5
0.3mM MEAP	132 ± 2	104 ± 1	131 ± 1	99 ± 2
1.0mM MEAP	134 ± 2	107 ± 1	132 ± 1	97 ± 1
Saline	134 ± 1	102 ± 2		
0.001mM NT	136 ± 2	97 ± 0.9	134 ± 1	101 ± 2
0.02mM NT	137 ± 2	95 ± 1	135 ± 2	98 ± 3
0.07mM NT	135 ± 1	93 ± 0.8	136 ± 1	94 ± 2
0.22mM NT	136 ± 2	94 ± 1	135 ± 2	96 ± 2
Saline	135 ± 0.9	91 ± 3		
0.001mM Deltorphin	137 ± 2	90 ± 2	134 ± 1	90 ± 1
0.03mM Deltorphin	135 ± 2	92 ±2	133 ± 2	92 ± 1
0.1mM Deltorphin	131 ± 1	90 ± 2	130 ± 2	89 ± 2
0.3mM Deltorphin	131 ± 2	90 ± 2	132 ± 1	91 ± 2
1.0mM Deltorphin	129 ± 1	91 ± 2	130 ± 1	92 ± 1

There were no significant differences in baseline heart rate (HR) and mean arterial pressure (MAP) measurements, during the delta receptor studies (n = 25).

Table 4: Average Measurements from Kappa Receptor Studies

Nodal Perfusate			Baseline After Washout (with saline)	
	HR ± SE	MAP ± SE	HR ± SE	MAP ± SE
Saline	140 ± 2	99 ± 2		
0.01mM Dynorphin	143 ± 3	97 ± 1	139 ± 1	95 ± 2
0.03mM Dynorphin	143 ± 2	100 ± 1	137 ± 2	93 ± 1
0.1mM Dynorphin	140 ± 4	101 ± 3	141 ± 3	100 ± 2
0.3mM Dynorphin	143 ± 4	99 ± 1	138 ± 1	99 ± 2
1.0mM Dynorphin	136 ± 5	91 ± 4	134 ± 3	97 ± 5
Saline	137 ± 2	94 ± 2		
0.001mM norBNI	136 ± 5	91 ± 4	138 ± 1	95 ± 2
0.02mM norBNI	131 ± 3	95 ± 1	136 ± 2	99 ± 3
0.07mM norBNI	134 ± 2	93 ± 2	135 ± 1	96 ± 2
0.22mM norBNI	130 ± 6	89 ± 5	134 ± 2	96 ± 2
Saline	136 ± 2	94 ± 3		
0.001mM U50 488	135 ± 2	92 ± 2	135 ± 2	94 ± 2
0.03mM U50 488	135 ± 3	95 ±2	135 ± 3	93 ± 4
0.1mM U50 488	134 ± 2	94 ± 3	130 ± 5	90 ± 4
0.3mM U50 488	133 ± 4	92 ± 3	132 ± 2	92 ± 2
1.0mM U50 488	132 ± 3	92 ± 2	132 ± 6	93 ± 2

There were no statiscally significant differences in baseline heart rate (HR) and mean arterial pressure (MAP) measurements, during the kappa receptor studies (n = 15).

Table 5: Average Measurements from Mu Receptor Studies

Nodal Perfusate			Baseline After Washout (with saline)	
	HR ± SE	MAP ± SE	HR ± SE	MAP ± SE
Saline	138 ± 4	100 ± 4		
0.01mM Endomorphin	139 ± 3	98 ± 3	139 ± 1	96 ± 2
0.03mM Endomorphin	140 ± 1	100 ± 2	138 ± 1	95 ± 2
0.1mM Endomorphin	141 ± 3	101 ± 3	139 ± 3	100 ± 2
0.3mM Endomorphin	138 ± 4	99 ± 1	139 ± 1	100 ± 3
1.0mM Endomorphin	137 ± 6	95 ± 4	139 ± 3	99 ± 5
Saline	137 ± 2	94 ± 2		
0.001mM CTAP	138 ± 6	93 ± 5	140 ± 2	95 ± 3
0.02mM CTAP	139 ± 4	99 ± 5	143 ± 3	93 ± 4
0.07mM CTAP	139 ± 3	93 ± 5	137 ± 2	97 ± 3
0.22mM CTAP	134 ± 6	90 ± 5	135 ± 4	99 ± 3
Saline	137 ± 3	95 ± 3		
0.001mM Super DALDA	136 ± 3	96 ± 2	135 ± 4	94 ± 3
0.03mM Super DALDA	137 ± 4	97 ± 4	134 ± 4	95 ± 4
0.1mM Super DALDA	138 ± 3	99 ± 5	133 ± 5	92 ± 4
0.3mM Super DALDA	134 ± 4	94 ± 3	132 ± 5	94 ± 2
1.0mM Super DALDA	130 ± 5	99 ± 2	134 ± 6	95 ± 2

There were no statiscally significant differences in baseline heart rate (HR) and mean arterial pressure (MAP) measurements, during the mu receptor studies (n = 17).

Table 6: Heart Rate (HR) and Mean Arterial Pressure (MAP) Data from the Nodal Artery Occlusion Studies

	Dog #	Control		Leu-Arg		PC		15mO		15mR	
		HR	MAP	HR	MAP	HR	MAP	HR	MAP	HR	MAP
	3874	142	100	141	94	143	102	144	101	139	102
	3880	138	101	139	102	137	99	135	99	135	98
	3902	125	99	128	109	126	97	126	99	127	101
	3912	154	105	155	102	149	99	152	103	150	102
	3916	138	101	139	105	140	102	135	101	134	103
	3923	109	115	107	112	106	115	108	112	110	111
	3926	135	119	132	112	134	112	140	111	137	111
	3931	128	75	129	76	124	80	127	82	125	81
	3940	139	82	138	81	137	82	139	83	139	84
	4017	147	75	145	78	140	83	145	82	138	84
	4024	122	80	122	80	127	80	124	81	126	80
	3766	145	70	144	72	143	71	142	72	134	73
	3756	155	70	152	72	151	73	156	72	165	75
	3787	136	89	137	87	138	88	135	90	136	91
	3816	126	90	128	91	129	91	125	92	124	92
	3815	146	91	145	92	149	91	140	100	142	101
	3781	141	92	140	93	141	94	142	95	143	94
	3812	142	96	140	95	142	93	140	95	139	94
	3819	138	95	139	94	137	95	138	94	139	95
	3823	145	98	142	99	143	100	144	97	145	98
MEAN		138	92	137	92	137	92	137	93	136	94
SD		11	14	11	13	10	12	11	11	11	11
SE		0.6	0.7	0.5	0.6	0.5	0.6	0.5	0.6	0.6	0.5

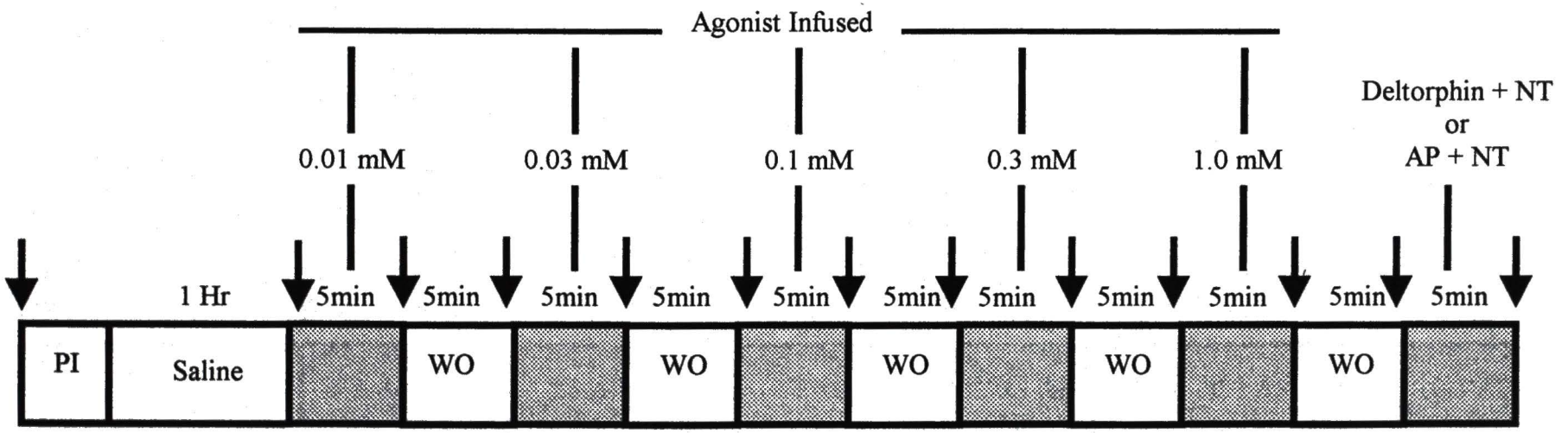
This table illustrates that there were no significant differences in baseline heart rate (HR) and mean arterial pressure (MAP) measurements during the low flow studies.

Table 7: Heart Rate (HR) and Mean Arterial Pressure (MAP) Data from the Nodal Artery Occlusion Studies (Extended Protocol II)

	Dog #	15mR		15mO		15mR		NT		Glib	
		HR	MAP	HR	MAP	HR	MAP	HR	MAP	HR	MAP
	3902	127	101	129	102	130	103	126	101	125	102
	3912	150	102	151	101	150	101	148	102	145	103
	3916	134	103	132	101	131	105	129	104	127	103
	3923	110	111	109	104	105	103	100	101	101	102
	3926	137	111	135	114	134	112	136	110	127	120
	3931	125	81	124	80	123	79	120	76	119	78
	3940	139	84	139	85	140	85	145	87	149	88
	4017	138	84	134	84	132	83	132	85	132	86
MEAN		133	97	132	96	131	96	130	96	128	98
SD		12	12	12	12	13	12	15	12	15	13
SE		1.5	1.5	1.5	1.5	1.6	1.5	1.9	1.5	1.9	1.6

This table illustrates that there were no significant differences in baseline hemodynamics during the extended protocol studies.

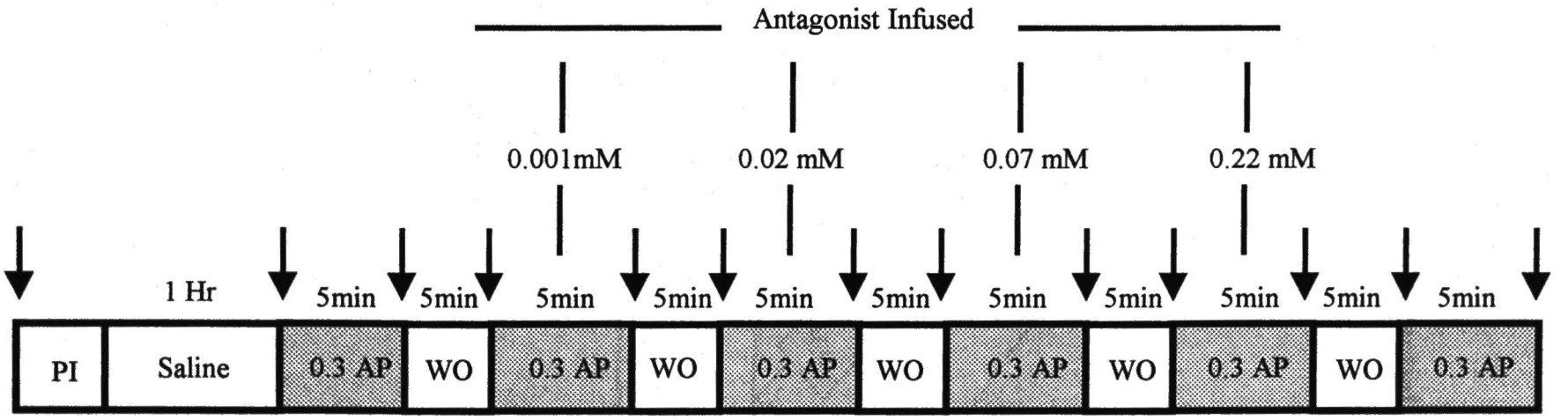
Illustration of Agonist Studies Protocols



Legend:

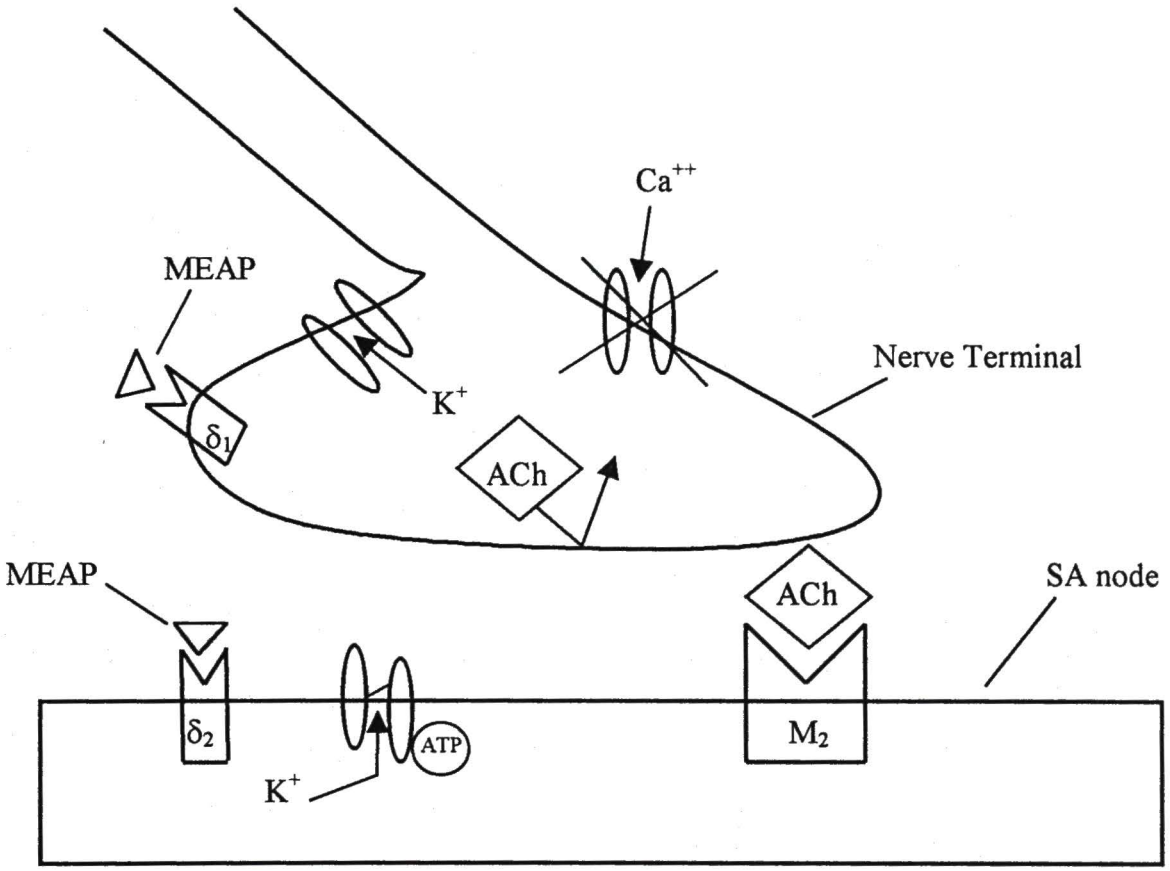
PI – Probe Inserted
WO – washout with saline
AP + NT – coinfusion of MEAP (0.3mM) and Naltrindole (0.07m)
↓ – 1, 2 and 3 Hz Stimulation of Right Vagus Nerve
Agonist – MEAP, Deltorphin, Endomorphin, Super DALDA, Dynorphin, or U50 488

Illustration of Antagonists Studies Protocols

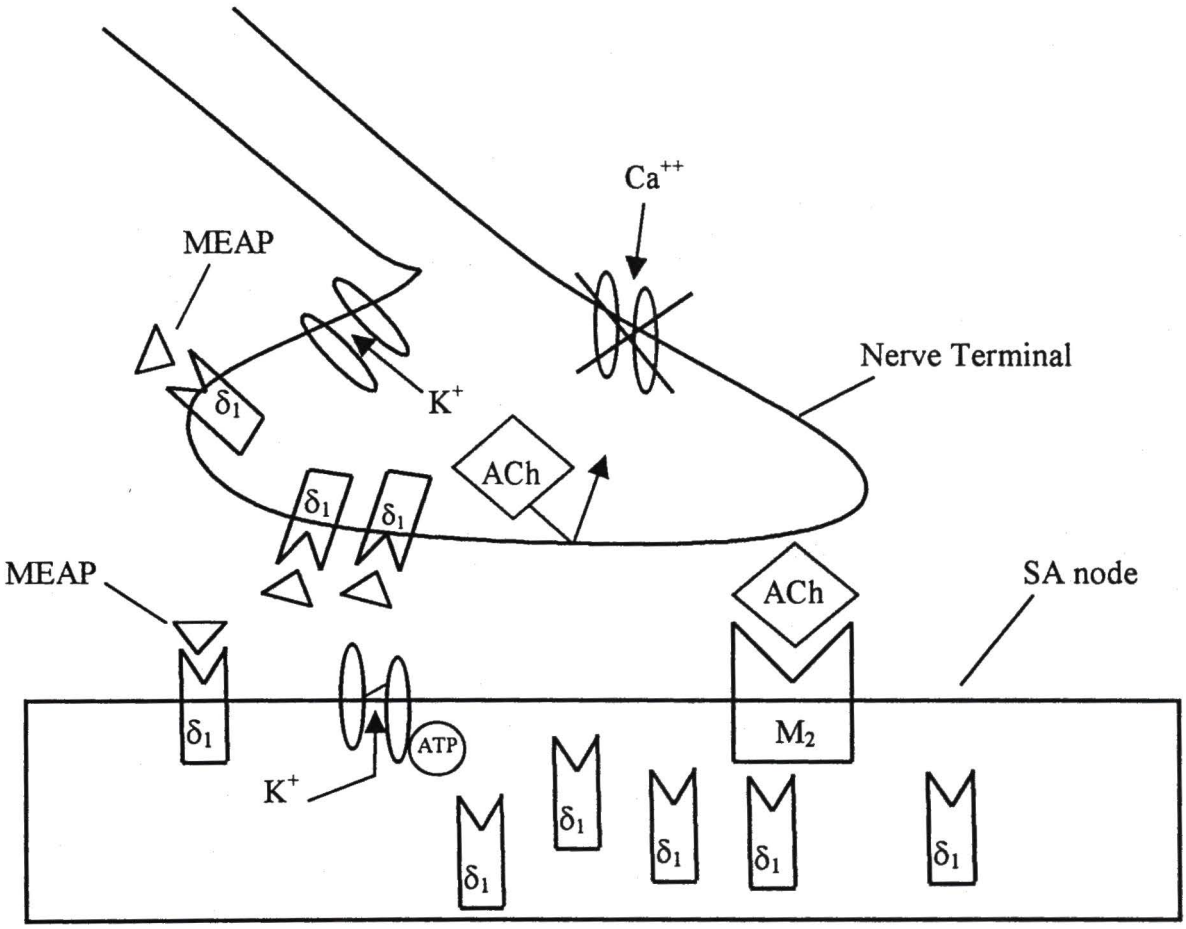


Legend: PI – Probe Inserted
0.03 AP – 0.03mM MEAP Infused
WO – washout with saline
↓ – 1, 2, 3 Hz Simulation of Right Vagus Nerve
Antagonist Infused – Naltrindole, CTAP, or norBNI

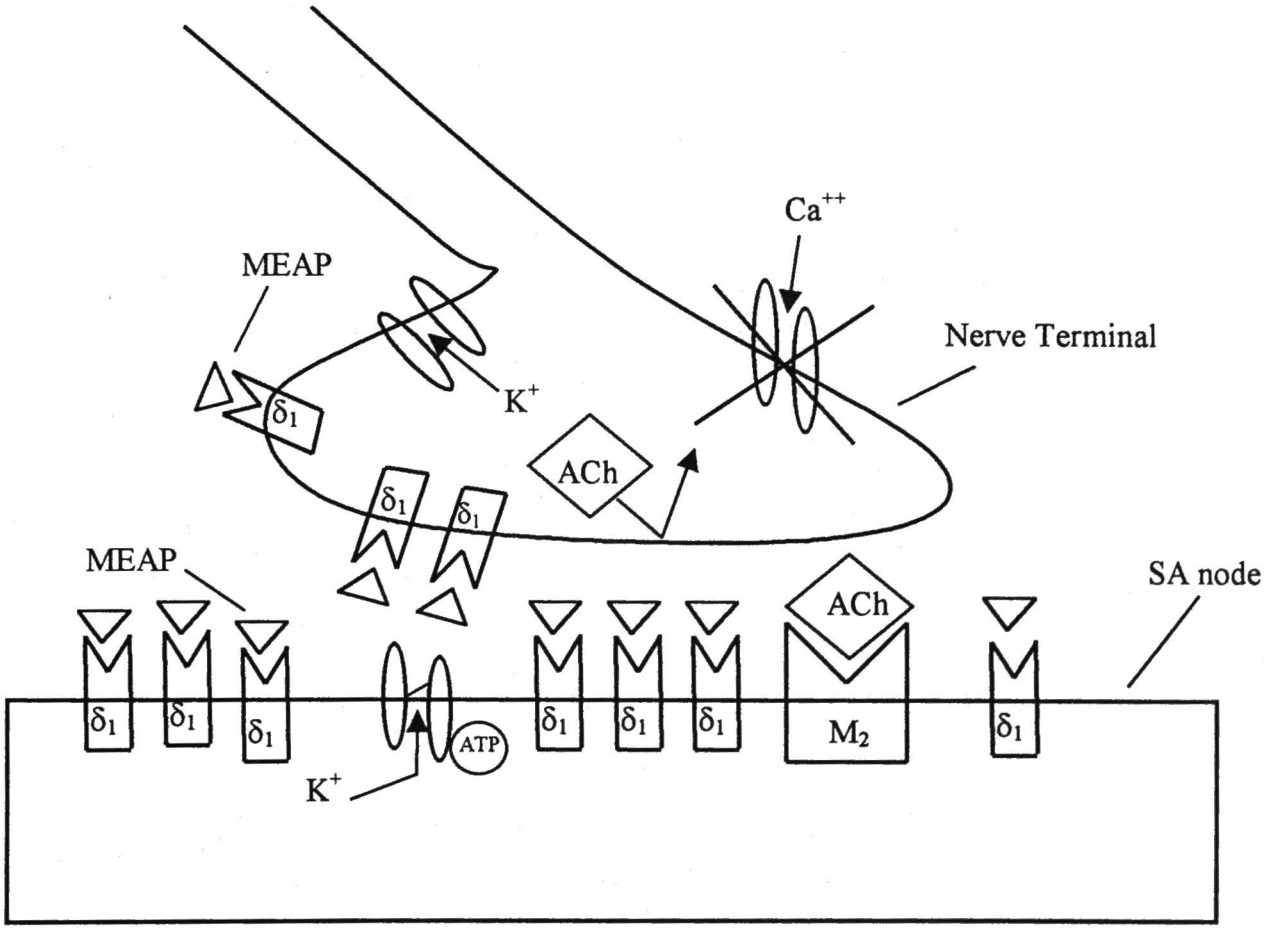
Differential Coupling



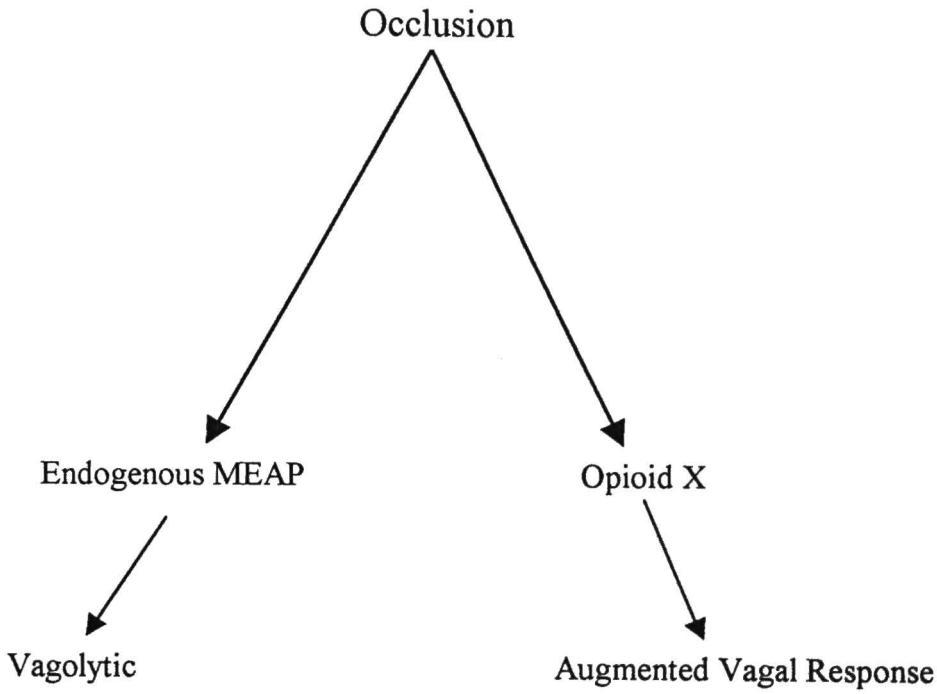
Differential Receptor Distribution
(Control)



Differential Receptor Distribution
(Low Flow)



Coincident but Unrelated Effects of Occlusion



Possible Therapeutic Actions of Potassium Channel Openers