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This study examined the hypothesis that the vagotonic and sympatholytic effects of cardiac enkephalins are independently mediated by different receptors. In study one, the heart rate response to increasing doses of the δ-receptor opioid, MEAP was determined during nerve stimulation. MEAP was administered by microdialysis into the interstitium of the canine sinoatrial node during sympathetic (right ansa subclavia) and parasympathetic (right cervical vagus) stimulation. This protocol was conducted to illustrate that the vagotonic effect of MEAP was independent of any simultaneous sympatholytic activity that might be intrinsic to MEAP. The right cardiac sympathetic nerves were isolated as they exited the stellate ganglion and were stimulated at frequencies selected to produce an intermediate increase in heart rate (HR) of approximately 35 bpm. The cervical vagi were ligated and the right vagus nerve was stimulated at frequencies selected to produce a two-step decline in heart rate of approximately 25 and 50 bpm. Dose response relationships were constructed by recording the change in heart rate during nerve stimulation as the dose of MEAP was increased in 5-min steps from 0.05 pmoles/min to 1500 pmoles/min. A significant increase in vagal transmission was observed during the administration of the δ-agonist, MEAP at 0.5 pmoles/min as evident by a greater decline in heart rate. The sympathetically mediated tachycardia was unaltered at this or any other dose of MEAP. In study two, a similar dose response relationship was constructed with the κ-opioid receptor agonist, U-50488H to illustrate an independent sympatholytic effect and to verify its κ-

receptor character. U-50488H gradually suppressed the sympathetic tachycardia with a significant effect obtained at the highest dose (1500 pmoles/min). U-50488H had no effect on vagally mediated decreases in heart rate. Surprisingly the sympatholytic effect of U-50488H was not reversed by withdrawal of the agonist or by addition of the κ-antagonist, norBNI. Study three was conducted to determine whether the sympatholytic effect to U-50488H could be prevented by co-administration of norBNI. NorBNI blocked the sympatholytic effect of the U-50488H throughout 90 min of exposure. When norBNI was discontinued after 90 min and U-50488H was continued alone, its sympatholytic effect reappeared within 30 min. Collectively these observations support the hypothesis that the vagotonic influence of MEAP was independent of sympathetic transmission and sympathetic transmission was unaltered by MEAP. Furthermore the observed sympatholytic effect of U-50488H was mediated independently by κ-receptors. The sympatholytic effect of sustained κ-receptor stimulation appears to evolve gradually into a functional state not easily reversed.

VAGOTONIC EFFECTS OF ENKEPHALIN

ARE NOT MEDIATED BY SYMPATHOLYTIC MECHANISMS

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PUBLICATIONS

- **Stanfill A, Jackson KE, Farias M, Barlow M, Deo S, Johnson S, Caffrey JL.** Leucine-Enkephalin interrupts sympathetically mediated tachycardia prejunctionally in the canine sinoatrial node. Proc Soc Experimental Biology and Medicine: 228(8): 898-906, 2003

- **Brooks WM, Stideley C, Petropoulos H, Yung RE, Weers D, Friedman SD, Barlow MA, Sibbitt WL Jr, Yeo RA.** Metabolic and cognitive response to human traumatic brain injury: A quantitative proton magnetic resonance study. Journal of Neurotrauma. 17(8):629-40 2000

Under Review

- **Vinogradoca TM, Lyashkov AE, Zhu W, Yang D, Deo S, Barlow MA, Johnson S, Caffrey JL, Zhou Y, Maltsev VA, Lakatta EG.** Pacemaker cells decode and translate protein kinase A signals into intrinsic rhythmic Ca2+ oscillations that regulate the heart's beating rate. **(submitted)**

- **Olivencia-Yarvarti AH, Mallet RT, Ortolana GA, Paul G, Barlow MA, Deo S, Daniel N, Johnson S, Caffrey JL.** Leukocyte filtration for off-pump coronary artery bypass. **(submitted)**

Abstracts

- **Barlow MA, Daniel N, Deo S, Johnson S, Yoshishige D, Caffrey JL.** Vagotonic effects of enkephalin are not mediated by sympatholytic mechanisms. 12th annual Research Appreciation Day) University of North Texas Health Science Center 2004

- **Caffrey JL, Deo S, Barlow MA, Johnson S, Farias M.** Opioid-Ganglioside interactions during vagal bradycardia. FASEB J. 18: A1074, 2004

- **Stanfill A, Jackson K, Farias M, Barlow M, Deo S, Johnson S, Caffrey JL.** Kappa-opioid receptors in the cardiac pacemaker decrease sympathetic tachycardia. Integrative Physiology/ Cardiovascular Research Institute UNTHSC, Fort Worth Texas. (11th annual Research Appreciation Day) University of North Texas Health Science Center 2003

- **Barlow MA, Petropoulos H, Kilgore D, Zamora LL, Hart BL, Sibbitt WL Jr, Brooks WM.** Reliability of assessing atrophy in Systemic Lupus Erythematosus. Proc Intl Soc Mag Reson Med 8: P 1242, 2000

- **Brooks WM, Stideley CA, Petropoulos H, Jung RE, Weers DC, Friedman SD, Barlow MA, Sibbitt WL Jr, Yeo RA.** Neuronal recovery following traumatic brain injury: 1H-MRS evidence in humans. Proc Intl Soc Mag Reson Med 8: p. 516, 2000

- **Barlow MA, Deo S, Johnson S, Caffrey JL.** Vagotonic effects of enkephalin are not mediated by sympatholytic mechanisms. FASEB, 2005 **(submitted)**

- **Johnson-Davis S, Deo S, Barlow MA, Yoshishige D, Caffrey JL.** GM-1, Deltorphin, and d-2 receptor plasticity in the SA Node. FASEB, 2005 **(submitted)**

- **Deo S, Barlow MA, Johnson S, Daniel N, Caffrey JL.** Repeated arterial occlusions improve vagal transmission in the sinoatrial node without eliminating the vagolytic response to opioids. FASEB, 2005 **(submitted)**

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CHAPTER I

INTRODUCTION

The electromechanical control of the heart is affected in large part by the two limbs of the autonomic nervous system. The sympathetic nervous system increases heart rate by releasing the neurotransmitter norepinephrine (NE) which then binds and activates postjunctional β-adrenergic receptors. The β-receptor activation induces a cascade of responses in the target cells mediated in part by the second messenger cyclic adenosine monophosphate (cAMP). Cyclic-AMP increases the frequency and magnitude of intracellular Ca^{2+} release in pacemaker cells thus increasing their spontaneous rhythm and the resulting heart rate. The parasympathetic nervous system can decrease the heart rate by releasing acetylcholine. Acetylcholine binds to muscarinic receptors on the pacemaker cells and diminishes the synthesis of cAMP. When cAMP is reduced in these cells, Ca^{2+} transients are less frequent and heart rate falls. In the sinoatrial node (SA) this interaction between the sympathetic and parasympathetic nerve traffic determines rhythmic responses to changing circulatory demands. In order to maintain appropriate responses under diverse conditions, modulation of neurotransmitter release is required to assure orderly transitions and to prevent electrical rhythm disturbances that may cause myocardial damage or even death. Local modulation of heart rhythms by cardiac opioids represents an active area of current research.

Opioid peptides are hormones and neurotransmitters with pharmacological properties similar to opium. Extracts of the opium poppy, *Papaver somniferum* provide a large number of medicinally active pharmaceuticals including historically important analgesics

and antidiarrheals. The primary endogenous opioids are intimately involved with the autonomic control of the circulation and in this regard a recent study of chronically administered morphine in dog found both vagal and sympathetic mechanisms were altered (1). Although morphine is clearly an important pharmaceutical, its addictive properties and abusive potential have severely limited its therapeutic use.

Endogenous opioids comprise a group of related peptides that function as neuromodulators in a variety of biological systems including the heart. Opioid peptide families consist of enkephalins, dynorphins, endorphins, and endomorphins. Cardiac tissue and isolated cardiac myocytes contain dynorphins and enkephalins as well as the mRNA for their respective precursors prodynorphin and proenkephalin (2, 3, 4, 5). The production of opioids can be triggered by myocardial infarction (6, 7), aging (8), hypertension (9), and heart transplantation (10). The processing of proenkephalin requires selective hydrolysis at pairs of basic amino acids surrounding each biologically active sequence. Proenkephalin contains four different opioid sequences including four copies of methionine enkephalin (ME) and one each of leucine enkephalin (LE), methionine-enkephalin-arg-phe (MEAP), and methionine-enkephalin-arg-gly-leu. A past study found that the pentapeptides LE and ME had a common tetrapeptide sequence, Tyr-Gly-Gly-Phe- with either leucine or methionine at the C terminus (11). Once these opioids are processed into their active state, their half-life is determined by multiple peptidases (12).

In the heart three different functional opiate receptors have been identified: mu (μ), delta (δ), and kappa (κ). The functional characterization of opioid receptors was primarily conducted in neural systems and each class of receptor is identified by its distinct agonist/antagonist profile (13). The receptors bind opioid ligands at selective recognition sites to trigger specific biological responses. These responses are generally mediated by increasing potassium efflux, decreasing calcium influx or by inhibition of adenylyl cyclase. The change in potassium efflux hyperpolarizes neurons and the reduction in calcium slows the vesicular transport of neurotransmitters. Cyclic-AMP regulates a large variety of cellular responses but in nerves it facilitates neurotransmitter release. The opioid receptors interact with GTP binding proteins and thus are part of the larger family of G-protein coupled receptors. Contrary to the conventional inhibitory view, opioid receptor coupling is diverse and may include both inhibitory and excitatory responses (14).

Opioid receptor distribution in the heart is important since the opioid peptides appear to function as potent regulators of heart rhythm. The enkephalins in particular have been localized in the autonomic nerves and their ganglia and are thus well positioned to moderate heart rate (5). Opioid receptors within the SA node can interrupt or facilitate vagal transmission (15, 16, 17, 18). These opiate receptors are most likely located pre-junctionally on vagal nerve endings where they can control the release of acetylcholine (19). Previous reports have demonstrated when exogenous opioids were administered systemically or by microdialysis directly into the nodal interstitium, they inhibited vagally induced bradycardia (20, 21). However, the effect of the enkephalins on the

vagal response was later identified as bimodal (figure 1). Ultra low doses of the enkephalin, MEAP actually increased vagal bradycardia (15, 16). These two studies by Farias et al suggested that the improved vagal bradycardia was mediated by activation of a δ-1 opioid receptor, since the vagotonic response was duplicated by the δ-1 agonist Tan-67. This conclusion was verified when the response was blocked by a low-dose administration of the δ-1 antagonist BNTX. Interestingly, opioid-mediated preconditioning also appears to involve δ-1 receptors in that the δ-1 agonist Tan-67 preconditions the heart and preconditioning is abrogated by BNTX (22, 23). The bimodal effects of the δ-receptors indicate a spectrum of potential roles in regulating the heart at rest and during stress.

Activation of κ-opioid receptors in the heart alters the cardiac function *in vivo* and *in vitro* (24). These observations suggested that endogenous κ-opioid peptides could also regulate cardiac function. The sympathetic nerves of the heart mediate their action by the release of norepinephrine (NE). When circulatory demand increases, sympathetic release of NE adjusts the cardiac output by increasing the heart rate and stroke volume to meet the increased demand for oxygen. Intracoronary administration of the κ-selective opioid, dynorphin reduced contractile activity during sympathetic stimulation by suppressing the release of NE (2, 19, 25). Prodynorphin mRNA was recently detected in both the atrial and ventricular myocytes suggesting that cardiac κ-receptors may be regulated by dynorphins locally synthesized in cardiomyocytes (26, 27). Similar to δ-opioid receptors, κ-opioid receptor activation reduces infarct size during ischemia (7). Similarities of the

two opioid systems suggest that cross-talk between these receptor classes may produce cooperative or interactive responses.

Rationale

LE administered into the canine sinoatrial node interstitium by microdialyisis reduced tachycardia during electrical stimulation of the cardiac sympathetic nerves (28). The sympatholytic effect was not reversed by the δ-antagonist naltrindol indicating a non-δ-opioid receptor mechanism (figure 2). The LE effect was reversed using the κ-antagonist norBNI suggesting κ-opioid receptors were activated (figure 3). LE at this dose also produced a clear delta-mediated vagolytic effect (figure 4) similar to MEAP (15). Cardiac LE concentrations are quite low; thus it is likely that the sympatholytic effect is normally mediated by a more potent κ-agonist like dynorphin. If there is a sympatholytic enkephalin, the much more abundant MEAP would seem a better candidate. In fact the vagotonic effect of MEAP might be explained by the elimination of opposing sympathetic tachycardia. Therefore, the current study was designed to test the hypothesis that the vagotonic influence of MEAP is the result of reduced tachycardia secondary to a coincident sympatholytic effect. Secondly, this study was designed to verify the kappa character of the sympatholytic effect and to test whether a selective κ-agonist would produce a similar, more potent, or more efficacious sympatholytic effect than LE. Additionally, the study tested whether the sympatholytic effect of the κ-agonist U-50488H indirectly altered vagal function by withdrawal of sympathetic opposition.

SPECIFIC AIMS

1. Test the hypothesis that the vagotonic effect of MEAP on heart rate is the result of a reduction in tachycardia secondary to a coincident sympatholytic effect.
2. Test the κ-receptor character of the sympatholytic effect.
3. Test whether a κ-opioid receptor with mediated sympatholytic effect indirectly alters vagal function.
4. Test if a selective κ-agonist would produce a similar, more potent or more efficacious sympatholytic effect than LE.

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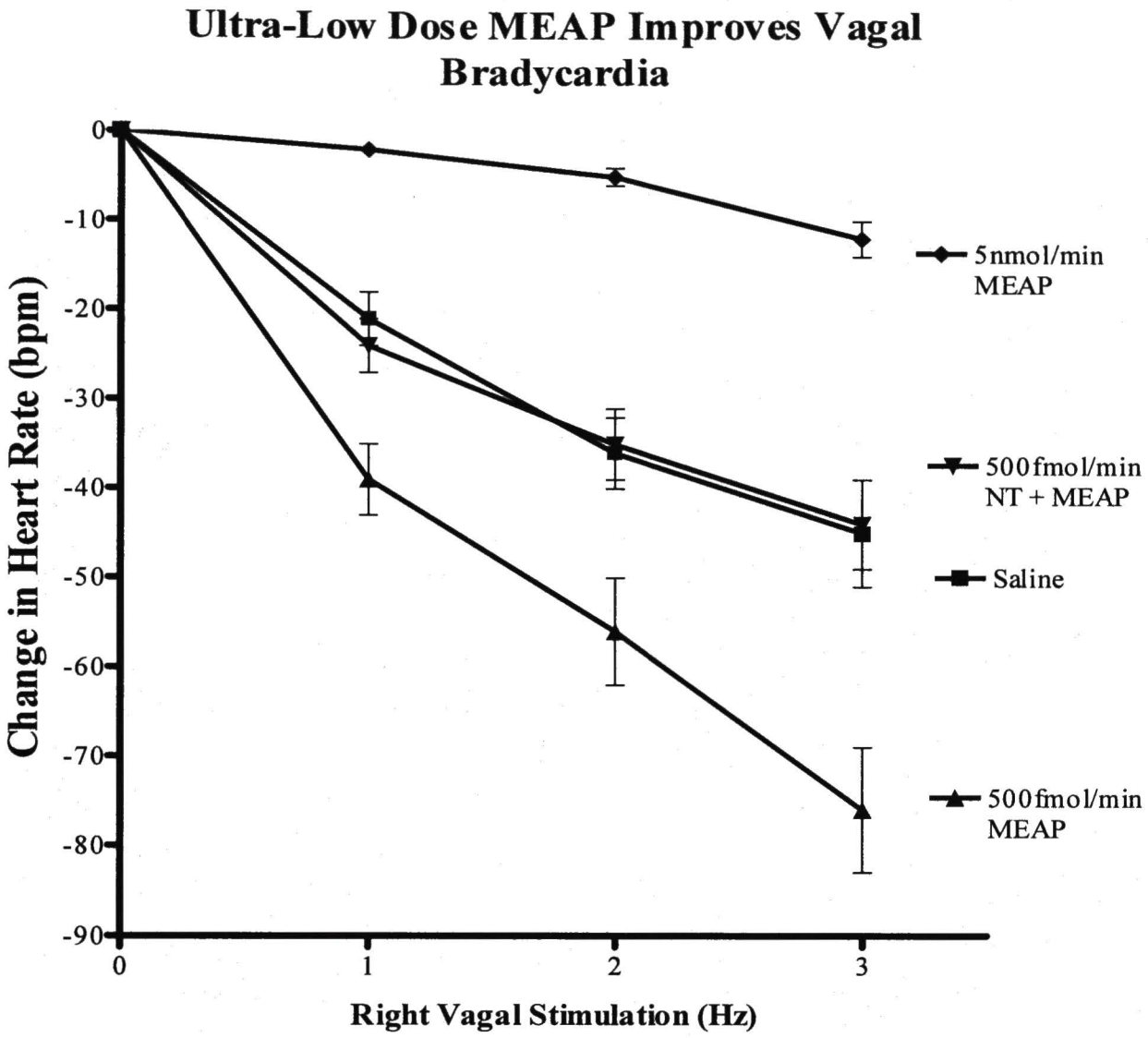

Figure 1. The graph illustrates the vagotonic effect of low doses of MEAP (lower curve) on heart rate/frequency response relationships during right vagal stimulation and their reversal when combined with low doses of the δ-antagonist, naltrindole (NT). In this example, the dose rates for MEAP and naltrindole were 500 fmoles/min. The figure also illustrates the vagolytic effect of higher dose rates (5 nmoles/min) in the same animals. The individual values represent the mean and S.E.; n = 5. The upper and lower curves are significantly different from control (15).

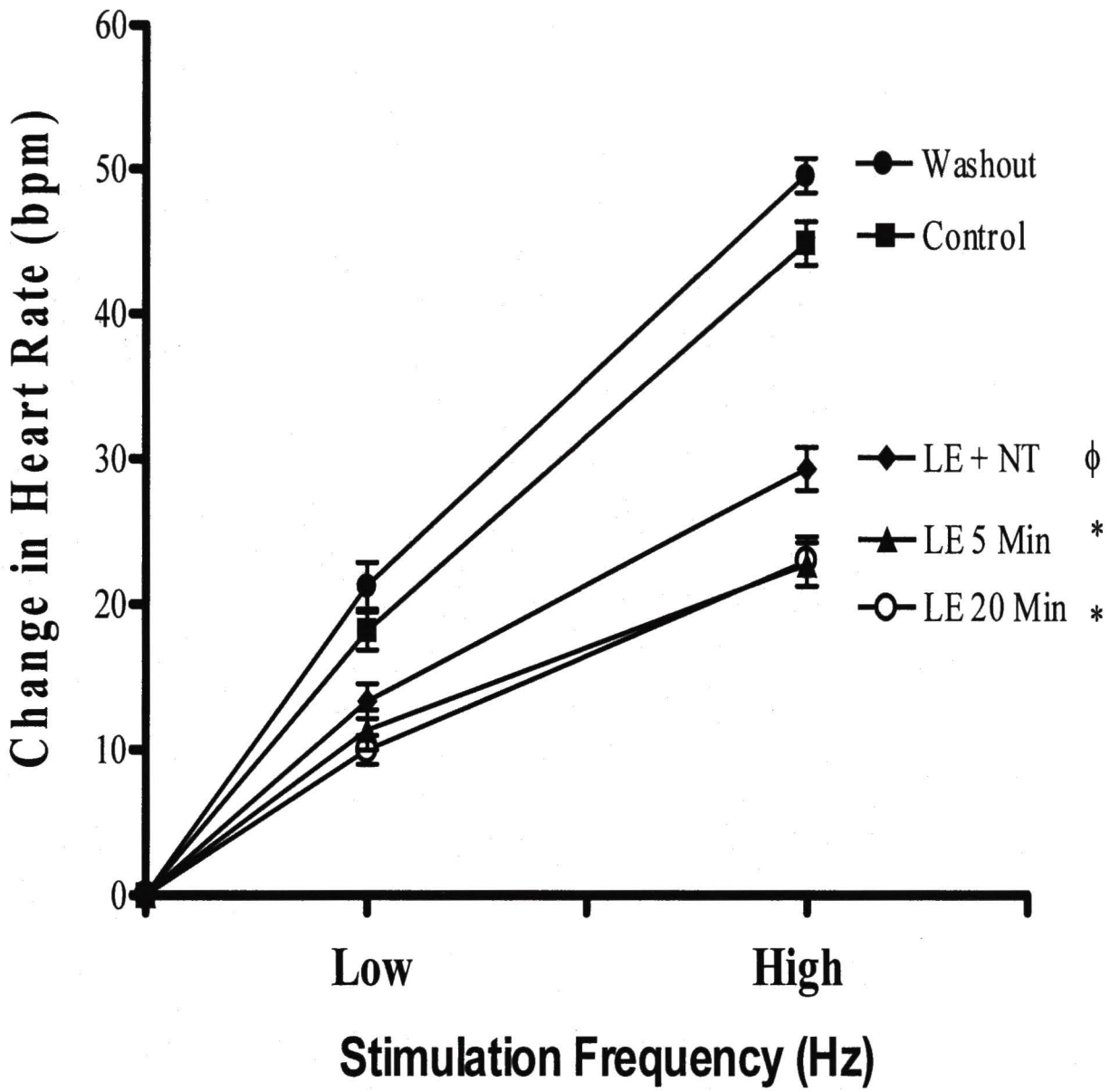

Figure 2: Changes in heart rate mediated by sympathetic stimulation are illustrated during nodal delivery by microdialysis of vehicle (■), leucine-enkephalin (LE) at 5 min (>), LE at 20 min (O), LE + naltrindole (LE + NT) (◆), and after washout of node with vehicle (●). Values are means and standard errors from 5 subjects. Sympathetic stimulation during LE infusion was significant reduced compared to control, $P < 0.05$ (✱). Sympathetic stimulation plus LE plus naltrindole was significantly different from both vehicle and leucine enkephalin (Φ, 28).

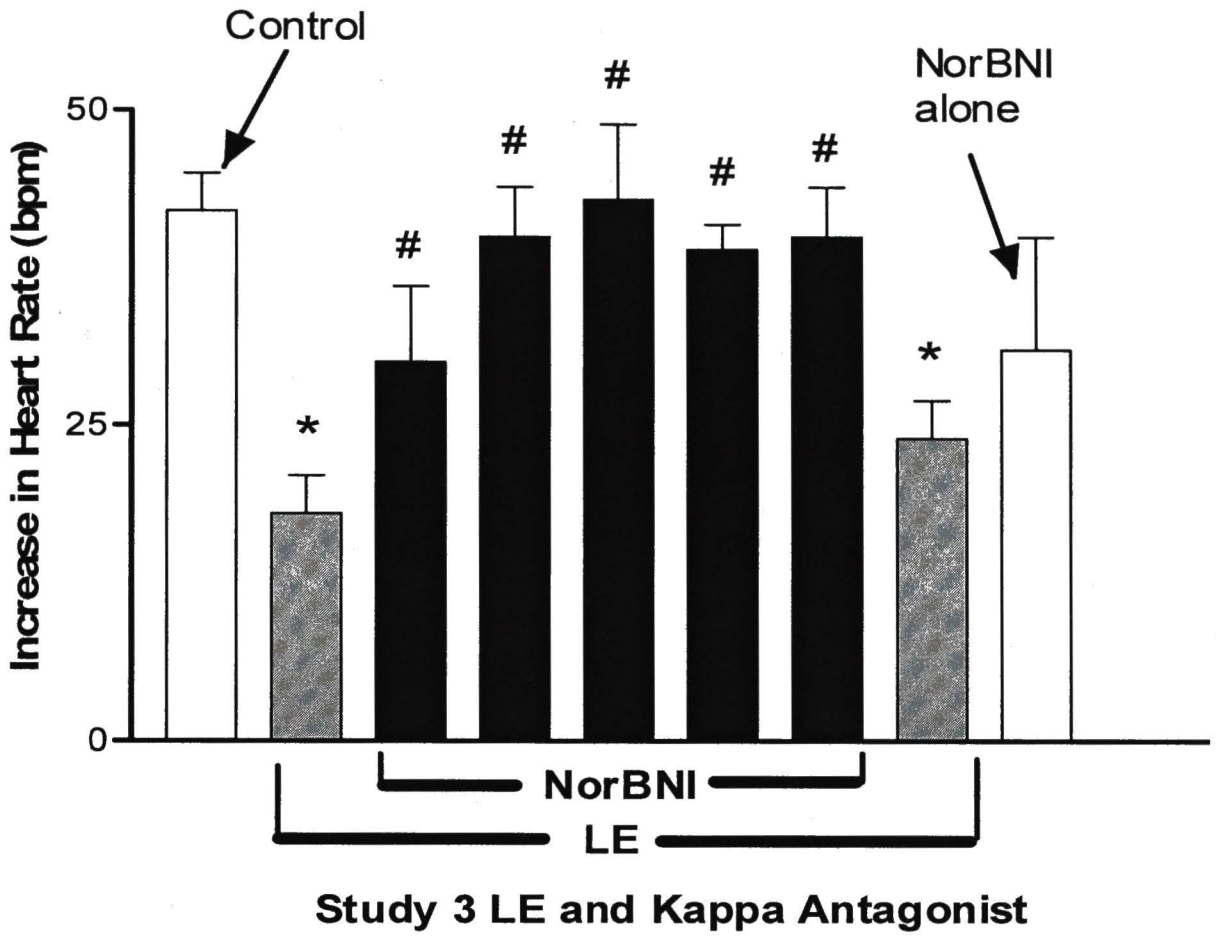

Figure 3: Changes in heart rate mediated by sympathetic stimulation are illustrated during nodal delivery of vehicle (control), leucine–enkephalin (LE, gray bars), LE + norbinaltorphimine (norBNI, black bars), or norBNI alone by microdialysis. A constant dose of LE (1.5 nmoles/min) was combined with step increases in the κ-antagonist, norBNI (0.01, 0.03, 0.1, 0.3, and 1 nmoles/min). After washout, the LE (1.5 nmoles/min) and norBNI (1.0 nmoles/min) were each retested alone as functional time and treatment

controls respectively. Values are means and standard errors from 5 subjects. Sympathetic stimulation during LE infusion was significantly reduced compared to control, $P < 0.05$ (✱). NorBNI reversed the LE mediated sympatholytic effect with an apparent ID_{50} of 0.01 nmoles/min. The symbol (#) indicates norBNI + LE combinations that were significantly different from LE, $P < 0.05$ (28).

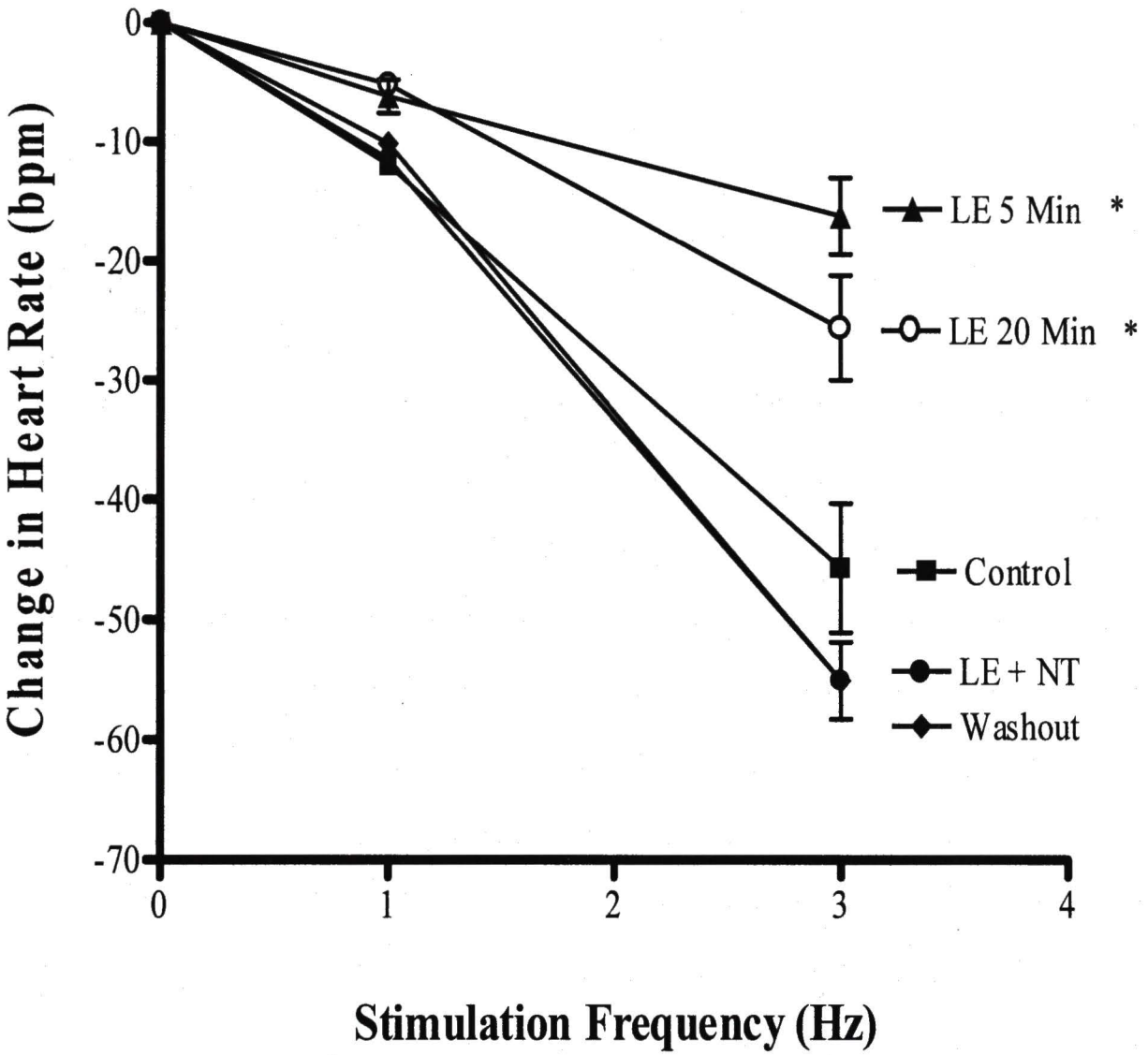

Figure 4: Changes in heart rate mediated by vagal stimulation are illustrated during the nodal delivery by microdialysis of vehicle (■), during Leucine-enkephalin (LE) at 5 min (▲), during LE at 20 min (⊙), during LE + naltrindole (LE+NT, ●), and after washout of node with vehicle (◆). Values are means and standard errors from 5 subjects. Vagal stimulation during LE infusion was significantly different from (✱=$P<0.01$) control (28).

CHAPTER II

VAGOTONIC EFFECTS OF ENKEPHALIN ARE NOT MEDIATED BY SYMPATHOLYTIC MECHANISMS

ABSTRACT

This study examined the hypothesis that the vagotonic and sympatholytic effects of cardiac enkephalins are independently mediated by different receptors. In study one, the heart rate response to increasing doses of the δ-receptor opioid, MEAP was determined during nerve stimulation. MEAP was administered by microdialysis into the interstitium of the canine sinoatrial node during sympathetic (right ansa subclavia) and parasympathetic (right cervical vagus) stimulation. This protocol was conducted to illustrate that the vagotonic effect of MEAP was independent of any simultaneous sympatholytic activity that might be intrinsic to MEAP. The right cardiac sympathetic nerves were isolated as they exited the stellate ganglion and were stimulated at frequencies selected to produce an intermediate increase in heart rate (HR) of approximately 35 bpm. The cervical vagi were ligated and the right vagus nerve was stimulated at frequencies selected to produce a two-step decline in heart rate of approximately 25 and 50 bpm. Dose response relationships were constructed by recording the change in heart rate during nerve stimulation as the dose of MEAP was increased in 5-min steps from 0.05 pmoles/min to 1500 pmoles/min. A significant increase in vagal transmission was observed during the administration of the δ-agonist, MEAP at 0.5 pmoles/min as evident by a greater decline in heart rate. The sympathetically mediated tachycardia was unaltered at this or any other dose of MEAP. In study two, a similar dose response relationship was constructed with the κ-opioid receptor agonist, U-50488H to illustrate an independent sympatholytic effect and to verify its κ-

receptor character. U-50488H gradually suppressed the sympathetic tachycardia with a significant effect obtained at the highest dose (1500 pmoles/min). U-50488H had no effect on vagally mediated decreases in heart rate. Surprisingly the sympatholytic effect of U-50488H was not reversed by withdrawal of the agonist or by addition of the κ-antagonist, norBNI. Study three was conducted to determine whether the sympatholytic effect to U-50488H could be prevented by co-administration of norBNI. NorBNI blocked the sympatholytic effect of the U-50488H throughout 90 min of exposure. When norBNI was discontinued after 90 min and U-50488H was continued alone, its sympatholytic effect reappeared within 30 min. Collectively these observations support the hypothesis that the vagotonic influence of MEAP was independent of sympathetic transmission and sympathetic transmission was unaltered by MEAP. Furthermore the observed sympatholytic effect of U-50488H was mediated independently by κ-receptors. The sympatholytic effect of sustained κ-receptor stimulation appears to evolve gradually into a functional state not easily reversed.

INTRODUCTION

Endogenous opioids comprise a group of peptides that function as neuromodulators in a wide variety of biological systems including the heart. Opioid peptide families consist of enkephalins, dynorphins, endorphins, and endomorphins. Cardiac tissue and isolated cardiac myocytes contain both dynorphins and enkephalins and the mRNA for their respective precursors, preprodynorphin and preproenkephalin (1, 2, 3, 4). The three classes of opioid receptors, μ,δ and κ have also been reported in heart (5). Although the mRNA for the precursor peptides was easily demonstrated (3, 6, 7, 8) the yield of receptor mRNA was limited. This disparity is consistent with a neuromodulatory function for the peptides and a presumed primary location for cardiac opioid receptors on cardiac neurons.

Cardiac opioids are elevated in neonatal animals, decline during adulthood and accumulate again late in life (3, 7, 8). They are acutely elevated after myocardial infarction, autonomic denervation (2, 7, 8) and during hemorrhagic hypotension (9). Circulating and/or myocardial opioids are chronically elevated in genetic hypertension, cardiomyopathic hearts, congestive heart failure and heart transplantation (10, 11, 12, 13, 14, 15). Cardiac opioids and their receptors have recently become therapeutically interesting because they have been implicated in protecting the heart from reperfusion injury, prevention of subsequent arrhythmias, and in extending the viable lifespan of harvested tissues (16, 17, 18). Less is known about the physiological function of opioids in heart but as neuromodulators, their interaction with cardiac autonomic innervation has become a productive area of inquiry. This study specifically focused on the paracrine

interactions between locally administered opioids and the autonomic innervation of the cardiac pacemaker.

Myocardial opioid receptors and their distribution is important since the opioid peptides appear to function as potent regulators of heart rhythm. Though enkephalins can originate in cardiomyocytes, they were also localized in the autonomic nerves and their ganglia including cardiac vagal structures and stellate ganglia where they are well positioned to moderate heart rate (19, 20, 21). Opioid receptors within the SA node can interrupt or facilitate vagal transmission (22, 23, 24, 25). When exogenous opioids were administered systemically or directly into the nodal interstitium by microdialysis, they inhibited vagally induced bradycardia (22, 26). These opiate receptors are apparently pre-junctional and most likely located on post-ganglionic vagal nerve endings where they can moderate the release of acetylcholine (27). The effect of the enkephalins on the vagal transmission was later identified as bimodal. Ultra low doses of the enkephalin, methionine-enkephalin-arginine-phenylalanine (MEAP) actually increased vagal bradycardia (28). The vagotonic effect was mediated by activation of a δ-1 opioid receptor, since the response was duplicated by the δ-1 agonist Tan-67 and reversed by the δ-1 antagonist BNTX. Interestingly, opioid-mediated preconditioning also appeared to involve δ-1 receptors in that the δ-1 agonist TAN-67 preconditioned the heart and ischemic preconditioning was abrogated by BNTX (25, 29). The bimodal effects of the δ-receptors indicate a spectrum of potential roles in regulating the heart at rest and during stress.

Activation of κ-opioid receptors in the heart also modifies cardiac activity *in vivo* and *in vitro* (30, 31, 32). Intracoronary administration of the κ-selective opioid, dynorphin reduced contractile activity during sympathetic stimulation by suppressing the release of NE (1, 27, 33). Furthermore the presence of prodynorphin mRNA in both atrial and ventricular myocytes suggested that cardiac κ-receptors may be activated by locally synthesized dynorphins (34, 35). Similar to δ-opioid receptors, κ-opioid receptor activation reduces infarct size during ischemia (30, 32). Similarities of the two opioid systems suggest that cross-talk between these receptor classes may produce cooperative or interactive responses.

Leucine enkephalin (LE) administered into the canine SA node by microdialysis reduced tachycardia during electrical stimulation of the cardiac sympathetic nerves (31). The sympatholytic effect was not reversed by the δ-antagonist naltrindole indicating a non-δ-opioid receptor mechanism. This LE effect was reversed by the κ-antagonist norBNI suggesting κ-opioid receptor participation. LE at this dose also produced a clear δ-medicated vagolytic effect similar to that of MEAP (28). Cardiac LE concentrations are quite low thus it seems likely that the sympatholytic effect is normally mediated by a more potent or more selective κ-agonist like dynorphin. If enkephalins are sympatholytic, the more abundant, MEAP would represent a better candidate. In fact, very low does of MEAP were vagotonic and this improved vagal transmission might be explained by the elimination of an opposing sympathetic tachycardia. Therefore the current study was designed to examine the hypothesis that the vagotonic and sympatholytic effects of cardiac enkephalins are independently mediated by different

receptors. More specifically the study tested the hypothesis that the vagotonic influence of MEAP was not the result of reduced tachycardia secondary to a coincident sympatholytic effect. The study was also constructed to verify the κ-receptor character of the sympatholytic effect and to test whether a selective κ-agonist would produce a similar, more potent and/or more efficacious sympatholytic effect.

General Surgical Preparation

22 Mongrel dogs of either gender weighing (15-30 kg) were assigned at random to the experimental protocols. Six subjects were excluded due to failure to complete the assigned protocol. 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* (NIH Publication No. 85-23, revised 1996). The animals were anesthetized with sodium pentobarbital (32.5 mg/kg), intubated and initially ventilated mechanically at 225 ml/min/kg with room air. Fluid filled catheters were 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 pressure transducer to measure heart rate and arterial blood pressure continuously on-line (PowerLab, ADI Instruments). The depth of anesthesia was regularly evaluated and supplemental anesthetic was administrated as required. The acid-base balance and blood gases were determined with an Instrumental Laboratories blood gas analyzer. The pO_2 (90-120 mmHg), the pH (7.35-7.45), and the pCO_2 (30-40 mmHg) were adjusted to normal by administering supplemental oxygen, bicarbonate or by modifying the tidal volume.

The right and left cervical vagus nerves were isolated from the carotid arteries through a ventral midline incision. The nerves were double ligated with umbilical tape to eliminate afferent vagal nerve traffic and reflex vagal compensation during vagal and sympathetic stimulation respectively. Anesthesia was carefully evaluated and a single dose of succinylcholine (50 μg/kg) was administered intravenously to reduce muscle movement

for the 10-min required for electrosurgical incision of the chest. A thoracotomy was performed to expose the right heart and the pericardium was opened and the pericardial margins were sutured to the body wall to support the heart. After the electrocautery was completed, the fluid filled arterial catheter was replaced with a high fidelity solid state transducer (Millar Instruments) to measure the arterial pressure and heart rate throughout the remainder of the protocol.

The sympathetic cardiac nerves distal to the right stellate ganglion (right ansa subclavia) were isolated and stimulated to determine the frequency (0.5-1.5 Hz) needed to produce a 30-40 bpm increase in heart rate during 45 sec of stimulation.

Nodal Microdialysis

The canine sinoatrial node can be located at the junction of the superior vena cava and the right atrium. A 25g stainless steel spinal needle containing the microdialysis line was inserted into the center of the sinoatrial node parallel to its long axis as previously described (23). The probe fabricated with dialysis fiber from a Clirans TAF 08 (Asahi Medical) artificial kidney was positioned with the dialysis window completely within the nodal tissue. The probe (1 cm x 200 μm ID x 220 μm OD) restricts the transmural passage of molecules with a mass greater than 36kD. The glass fiber inflow line was then attached to a micro infusion pump and perfused with vehicle (saline) at 5ul/min and allowed to equilibrate for 1 hr.

Protocol 1: *MEAP dose response during vagal and sympathetic stimulation.*

After equilibration for 1 hr, the right cervical vagus nerve was stimulated at a supra-maximal voltage (15 v) for 15 seconds at low (1-2 Hz) and high (3-4Hz) frequencies selected to produce respectively 10-20 bpm and 30-40 bpm decreases in heart rate. The system was allowed two minutes to recover between the successive stimuli. After an additional 2 min recovery, the sympathetic (right ansa subclavia) was stimulated at a frequency (1 Hz) selected to increase heart rate by 30 to 40 bpm. Once these control measurements were recorded a cumulative six-step MEAP dose response (0.05pmoles/min to1.5 nmoles/min) was initiated with the cumulative doses delivered in succession by dialysis. After 5 min exposure, the vagal/sympathetic stimulations were repeated and the next dose of MEAP was introduced. After completing the dose response, the dialysis perfusate was returned to saline for 10 minutes and the nerve functions were re-evaluated to verify complete recovery of control function and the absence of a time or treatment dependent change in basal nerve transmission.

Protocol 2: *U-50488H, (κ-agonist) response curve to vagal and sympathetic stimulation; test for reversal by norBNI (κ-antagonist).* The dose response described in protocol 1 was repeated while substituting the kappa agonist, trans-3,4-dichloro-N-methyl-N-(2-1-pyrrolidinyl)-cyclohexyl)cyclohexyl) benzenacetamide methanesufonate (U-50488H) for MEAP. After completing the dose response, the κ-agonist was discontinued and the probe was perfused with saline for 10 min. Nerve functions were re-evaluated to determine whether control values could be re-established. The κ-antagonist, 17,17'-(dichloropropylmethyl)-6,6',7,7'-6,6'-imino-7,7'-binorphinan-3,4',14,14'-

tetroldihydrochloride (norBNI) was perfused into the nodal probe at an equimolar dose rate to the maximal dose of U-50488H (1.5 nmoles/min) to test for antagonist effects independent of the added agonist. After 5 min, the stimulation sequence was repeated. NorBNI was washed out with saline for 20 min and the heart rate responses were evaluated once again to re-establish the control measurements and to wash the norBNI out of the nodal area. Finally, equimolar concentrations of U-50488H and norBNI were introduced together (1.5 nmoles/min each) to verify that the sympatholytic effect was mediated by a kappa receptor interaction.

Protocol 3: *NorBNI pretreatment prevents the sympatholytic effect of U-50488H*

This protocol was conducted to determine whether pretreatment with norBNI was more effective than adding it after sustained exposure to the agonist. The cervical vagus and thoracic sympathetic nerves were isolated as described above. After one hour of equilibration the sympathetic nerve was stimulated at 0.5 and 1 Hz for 45 sec each with 3 min of recovery between stimulations. The κ-antagonist norBNI (5 nmoles/min) was perfused alone in excess for 10 minutes followed by a second pair of sympatholytic stimulations. Then equimolar doses (1.5 nmoles/min) of norBNI and U-50488H were perfused for 90 minutes. The duration was selected to exceed the duration required to complete the previous dose response for U-50488H described in protocol 2. The nerve was tested every 30 minutes at each frequency. Following the stimulation at 90min, the norBNI was discontinued and U-50488H (1.5 nmoles/min) was continued alone for another 60 min. Additional nerve stimulations were tested every 15 minutes to monitor the emergence of the sympatholytic effect.

MATERIALS: MEAP was synthesized by American Peptide, in Sunnyvale, CA. NorBNI, Dynorphin A 1-13, and U-50488H were obtained from Sigma RBI in St. Louis, MO.

DATA ANALYSES: 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^{TM}, Dynamic Microsystems, Silver Springs, MD). Repeated measures comparisons were made where appropriate and $P < 0.05$ was accepted as a statistically significant difference.

RESULTS

The resting cardiovascular indices for all protocols are listed in Table 1. No differences were observed in the initial values for animals assigned to each protocol and there were no changes in resting function during any of the intra-nodal treatments.

Study 1: *MEAP improves parasympathetic transmission without modifying sympathetic transmission.* The lower and higher frequency vagal stimulation produced respectively mean decreases in heart rate of 20.4 $\pm$ 2.6 and 42 $\pm$ 8.1 bpm (Figure 1). MEAP produced a significant improvement in vagal transmission at 0.5 pmoles/min. The heart rate during low frequency stimulation was reduced by an additional 31% (29.6 $\pm$ 5.4 bpm). At the higher frequency the increase in enkephalin-mediated bradycardia was a less dramatic 10% (46.0 $\pm$7.4 bpm). At the higher end of the dose response (1.5 nmoles/min) the bimodal character of the response was evident and MEAP reduced the low frequency bradycardia by 62% (7.6 $\pm$ 2.1 bpm) compared to control. The high frequency response (18.5 $\pm$ 5.7 bpm) was similarly reduced by 55%. Both of the vagotonic and vagolytic effects were statistically different ($p<0.05$) from control and from each other. When the treatments were discontinued and the system was perfused again with vehicle, vagal stimulation produced bradycardia similar to the control. The restoration of control responses on washout also indicated that the changes in response to MEAP infusion were reversible and non-toxic. These observations were expected and confirmed findings from earlier studies (22, 23, 28).

After a 2-min recovery from each vagal stimulation sequence, the sympathetic nerves were stimulated for 45 seconds at 1 Hz. Sympathetic stimulation produced a mean increase of 38.8 ± 5.3 bpm (Figure 2). MEAP did not alter the rate of change in heart rate or the maximal tachycardia at any of the six dose rates. The inability of MEAP (a δ-agonist) to modify heart rate during sympathetic stimulation suggested that both the vagotonic and vagolytic effects of MEAP on vagal transmission are independent of sympathetic input and that unlike LE, MEAP had no intrinsic κ-receptor activity.

Study 2: The κ-agonist, U-50488H is sympatholytic

Study one indicated that MEAP at ultra low doses enhanced parasympathetically mediated bradycardia within the SA Node without altering the sympathetically induced tachycardia. In study two the κ-receptor opioid agonist, U-50488H was infused into the nodal interstitium to determine the role of nodal κ-receptors in sympathetic and parasympathetic mechanisms. Sympathetic stimulation increased heart rate an average of 41.2 ± 3.2 bpm (Figure 3). U-50488H produced a gradual decline in sympathetically mediated tachycardia starting at 50 pmoles/min and became significant at 1.5 nmoles/min. The resulting 32.7± 3.5 bpm represented a 25% reduction from control. Low and high frequency vagal stimulations reduced heart rate by 24.0 ± 3.9 and 45.0 ± 6.4 bpm respectively (Figure 4). Vagal bradycardia was unaltered by U-50488H. During infusion of the maximal dose of U-50488H, the vagal stimulation reduced heart rate by 28.4 ± 4.2 and 46.3 ± 7.1 bpm, which was not significantly different from control. The significant reduction in tachycardia during the administration of U-50488H indicated that

nodal κ-receptors influence sympathetic transmission independent of any vagal interaction.

Inconsistencies arose when the post-infusion evaluations were conducted. The sympatholytic effect remained in place even after 30 min washout with vehicle (35.5 ± 4.1). Furthermore, addition of the κ-antagonist (norBNI) for another 30-min also failed to reverse the sympatholytic effect (28.4±2.2). The sympathetic stimulation after norBNI was not significantly different from the maximally sympatholytic dose of U-50488H. Vagal evaluation at these same intervals indicated again no difference from control. In summary, the selective κ-agonist produced a clear sympatholytic response but the control response was not restored on washout or after administering a selective antagonist. The failure to restore normal sympathetic transmission suggested that exposure to U-50488H precipitated a very slowly resolving effect downstream from the κ-receptor or that the sympatholytic response was not mediated by κ-receptors. Study 3 was designed to differentiate between those alternatives.

Study 3: NorBNI prevents sympatholytic effect of U-50488H.

In this study, the κ-antagonist norBNI was infused into the SA Node by microdialysis prior to and concurrently with U-50488H to verify κ-receptor participation by preventing the subsequent sympatholytic effect of U-50488H. The sympathetic nerves were stimulated at lower (0.5 Hz) and higher (1 Hz) frequencies to produce average increases in heart rate of 29.3 ±6.2 and 62.0 ± 8.9 bpm respectively. During the initial 5-min infusion with excess norBNI alone (5 nmoles/min), the heart rate response to sympathetic

stimulation increased but was significant only at the lower frequency (Figure 5a). When U-50488H and norBNI were combined at 1.5 nmoles/min, no sympatholytic effect was observed throughout the 90-min period of infusion. When, norBNI was discontinued, and U-50488H was continued alone for an additional 60 minutes (Figure 5b), a sympatholytic effect gradually emerged. After 60 min, both low and high frequency responses were reduced respectively to 25.6 ± 6.4 and 43.7± 13.9 bpm.

DISCUSSION

The normal cardiac rhythms are primarily derived from interactions between the intrinsic rhythm of nodal pacemaker cells and incoming parasympathetic and sympathetic traffic. Changes in heart rate are quickly executed by increasing and decreasing efferent vagal transmission and less often by increasing sympathetic activity. As local neuromodulators, cardiac opioids are well positioned to modify these efferent autonomic influences as they innervate the pacemaker. The current study was prompted by the observation that enkephalins administered by dialysis into the SA node were both vagotonic and sympatholytic (28, 31). The first study was designed to test whether the vagotonic effect of MEAP was a direct effect on vagal transmission or an indirect consequence of a coincident sympatholytic influence. The result obtained demonstrated a clear vagotonic effect of MEAP in the complete absence of any complimentary sympathetic influence. Thus MEAP improves vagal transmission directly. This is consistent with the prior pharmacological evidence that the vagotonic effects were mediated by δ-1-receptors and the suggestion that the sympatholytic effects were mediated by κ-receptors (23, 28, 31, 33). Since MEAP did not alter sympathetic transmission, the vagotonic effect cannot be attributed to a reduction in sympathetic opposition. The failure of MEAP to alter sympathetic transmission suggests that unlike LE, which appears to have some κ-agonist activity in this system, MEAP had little or none regardless of the dose applied.

LE is an established δ-agonist and thus an unlikely candidate as the endogenous κ-agonist responsible for moderating sympathetic transmission. LE like MEAP is highly

vagolytic and like MEAP its vagolytic effect was eliminated by the δ-antagonist, naltrindole (31). The sympatholytic effect of LE that was reported earlier may reflect the higher dose used in that earlier study (31) and some of its efficacy at the κ-receptor may be attributed to its shared amino acid sequence with the native κ-agonist, dynorphin. The second study was designed to verify that the sympatholytic effect of LE could be more effectively duplicated by a more selective κ-agonist (36). Initial κ-agonist studies were conducted in several animals with the native κ-selective peptide, dynorphin A 1-13. Unfortunately dynorphin is highly charged and routinely adsorbs to surfaces. Despite attempts to prevent adsorption, there was no demonstrable effect of dynorphin on sympathetically mediated tachycardia. In the absence of a response, it is difficult to determine whether dynorphin was ineffective or simply never reached the target. As a result, the subsequent dose responses were conducted with the synthetic κ-agonist, U-50488H an alkaloid. As demonstrated in the second study, U-50488H was mildly sympatholytic. The reduction in sympathetic tachycardia appeared to emerge at lower doses but only reached significance at the highest dose. The full effect of sympathetic stimulation was not however restored when the U-50488H was washed out with vehicle and the sympatholytic effect was not reversed following the addition of the κ-antagonist norBNI. Though the data were not included, longer washouts and higher doses of the antagonist were evaluated in several animals without any change in the outcome. Thus it appears that U-50488H exerted a downstream effect that was either irreversible or only slowly reversible. Whether this was mediated by κ-receptors or through a non-κ-opioid-receptor mechanism required further confirmation.

Study three was thus designed to determine whether pretreatment with the κ-antagonist, norBNI would prevent the sympatholytic effect of U-50488H and confirm that the effect of U-50488H was mediated by interaction with κ-receptors. Surprisingly norBNI alone appeared to improve sympathetic transmission. Although this is suggestive of reversing the effect of an endogenous κ-agonist, it may also represent a direct effect of the initial loading dose of norBNI. The sympatholytic dose of U-50488H was then combined with norBNI for 90 min. The duration was selected to exceed the time required for the complete U-50488H dose responses in the previous protocol. NorBNI completely prevented the sympatholytic effect of U-50488H and the protection gradually eroded when the norBNI was discontinued. It is unclear whether the gradual onset reflects the temporal character of the κ-response or simply the time needed to washout the residual interstitial norBNI. Thus the sympatholytic effect of U-50488H was mediated by κ-opioid receptors. The resultant changes in function however may develop and resolve very slowly. In contrast, the sympatholytic effect of LE and the vagolytic effects of ME, LE, and MEAP all develop and resolve quickly (27,28, 31). Thus the reason for the slow κ-receptor kinetics is unclear but may be related to either the long duration of exposure during the dose response or may reflect the disposal characteristics of U-50488H in this specific model.

In summary the vagotonic effects of MEAP are independent of any coincident sympatholytic influences. They occur at very low dose rates and may represent a salutary compensation. Enkephalins accumulate following ischemic preconditioning and thus improved vagal transmission in the ischemic territory might reduce tissue injury by

reducing work and oxygen consumption locally in the tissue served by the occluded artery (37, 38). An additional sympatholytic effect to reduce myocardial oxygen demand further would be complimentary to the vagotonic effect of enkephalin. Locally sympatholytic κ-receptors thus might explain the cardioprotective activity reported following the introduction of κ-opioids prior to ischemia (30, 32). Based on low myocardial opioid LE content (1, 7, 26, 39) and on the dose response data presented above for MEAP, it seems unlikely that enkephalins would be responsible for a κ-mediated cardioprotective effect unless, for instance, LE was preferentially concentrated in close proximity to sympathetic neurons. The myocardial content of the prototypical endogenous κ-agonist, dynorphin is also limited, but its selectivity and greater potency may make dynorphin a better candidate for the regulating local sympathetic activity in heart (34, 35). The sympatholytic effect of U-50488H was less potent than expected though it was clearly mediated by κ-receptor activation. The magnitude of the response to U-50488H was limited and appeared to resolve very slowly if at all. Thus the κ-receptor response seems more suited to long term or background modulation.

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LEGENDS

Figure 1: Changes in heart rate mediated by low (1-2 Hz) and high (3-4 Hz) frequency stimulation of the right vagus nerve are illustrated during exposure to increasing doses of MEAP introduced into the interstitium of the SA node by microdialysis. Values are means and standard error of the mean from six subjects. The three symbols indicate that the change in heart rate was greater (#) than control, less (*) than control or less (†) than the peak vagotonic response $p<0.05$.

Figure 2: Changes in heart rate mediated by stimulation (1 Hz) of the right thoracic sympathetic nerves are illustrated during exposure to increasing doses of MEAP introduced into the interstitium of the SA node by microdialysis. Values are means and standard error of the mean from six subjects. No treatment effects were observed.

Figure 3: Changes in heart rate mediated by stimulation (1 Hz) of the thoracic sympathetic nerves are illustrated during exposure to increasing doses of the κ-agonist, U-50488H introduced into the interstitium of the SA node by microdialysis. Values are means and standard error of the mean from seven subjects. The symbol (*) indicates the change in heart rate was significantly different from control ($P < 0.05$).

Figure 4: Changes in heart rate mediated by low (1-2 Hz) and high (3-4 Hz) frequency stimulation of the right vagus nerve are illustrated during exposure to increasing doses of the κ-agonist, U-50488H introduced into the interstitium of the SA node by

microdialysis. Values are means and standard error of the mean from seven subjects. No treatment effects were observed.

Figure 5: (Panel A) Changes in heart rate mediated by lower (0.5 Hz) and higher (1 Hz) frequency stimulation of the right thoracic sympathetic nerves are illustrated during 5 min exposure to excess norBNI alone (5 nmoles/min) followed by 90 min of norBNI combined with U-50488H at equimolar dose rates (1.5 nmoles/min) introduced into the interstitium of the SA node by microdialysis. (Panel B) After 90 min the norBNI was discontinued and the U-50488H was continued alone for 60-minute. Values are means and standard error of the mean from three subjects. In Panel A the symbol (*) indicates that the change in heart rate was greater (*) than control ($P < 0.05$). In Panel B, the symbols indicate that the change in heart rate was less than control (†), less than at 90 min and control (#), $P<0.05$.

Table 1. Resting Heart Rate (HR) and Mean Arterial Pressure (MAP) Before Nerve Stimulation

		MEAP (pmoles/min)						
		Control	0.05	0.5	5	50	500	1500
Study One n=6	HR (bpm)	111.4 ±4.4	113 ±5.4	109.2 ±4.9	107.4 ±5.9	108.4 ±6.1	107.4 ±6.3	107.4 ±4.9
	MAP (mmHG)	96.2 ±2.4	96.4 ±1.9	98.8 ±1.7	97.1 ±2.8	98.6 ±2.2	98.2 ±2.3	98.8 ±2.4

		U-50488H (pmoles/min)						
		Control	0.05	0.5	5	50	500	1500
Study Two n=7	HR (bpm)	109 ±1.4	108.8 ±1.5	109.5 ±1.5	108.5 ±2.4	105.3 ±2.7	102.3 ±1.7	100.2 ±2.2
	MAP (mmHG)	93.5 ±3.2	93.8 ±3.8	94.7 ±4.3	95.3 ±3.9	96.3 ±3.7	96.0 ±3.2	96.2 ±3.2

				U-50488H (1.5 nmoles/min)				
norBNI		-	++	+	-	-	-	-
		Control	5 min	90min	15min	30min	45min	60min
Study Three n=3	HR (bpm)	108.7 ±8.3	107 ±6.8	101.7 ±4.4	99.0 ±6.7	94.3 ±10.4	95.0 ±9.2	94.7 ±9.8
	MAP (mmHG)	98.0 ±4.4	98.7 ±3.1	96.7 ±1.9	97.7 ±1.9	98.3 ±1.2	98 ±2.1	100.7 ±0.9

The values are expressed as means ± SE. In the three studies there was no significant effect of the δ-agonist, MEAP, the κ-agonist, U-50488H, or the κ-antagonist, norBNI, on resting HR and MAP. In study three, the symbols in the norBNI row indicate (- = none; + = 1.5 nmoles/min; and ++ = 5 nmoles/min) infusion of norBNI by microdialysis.

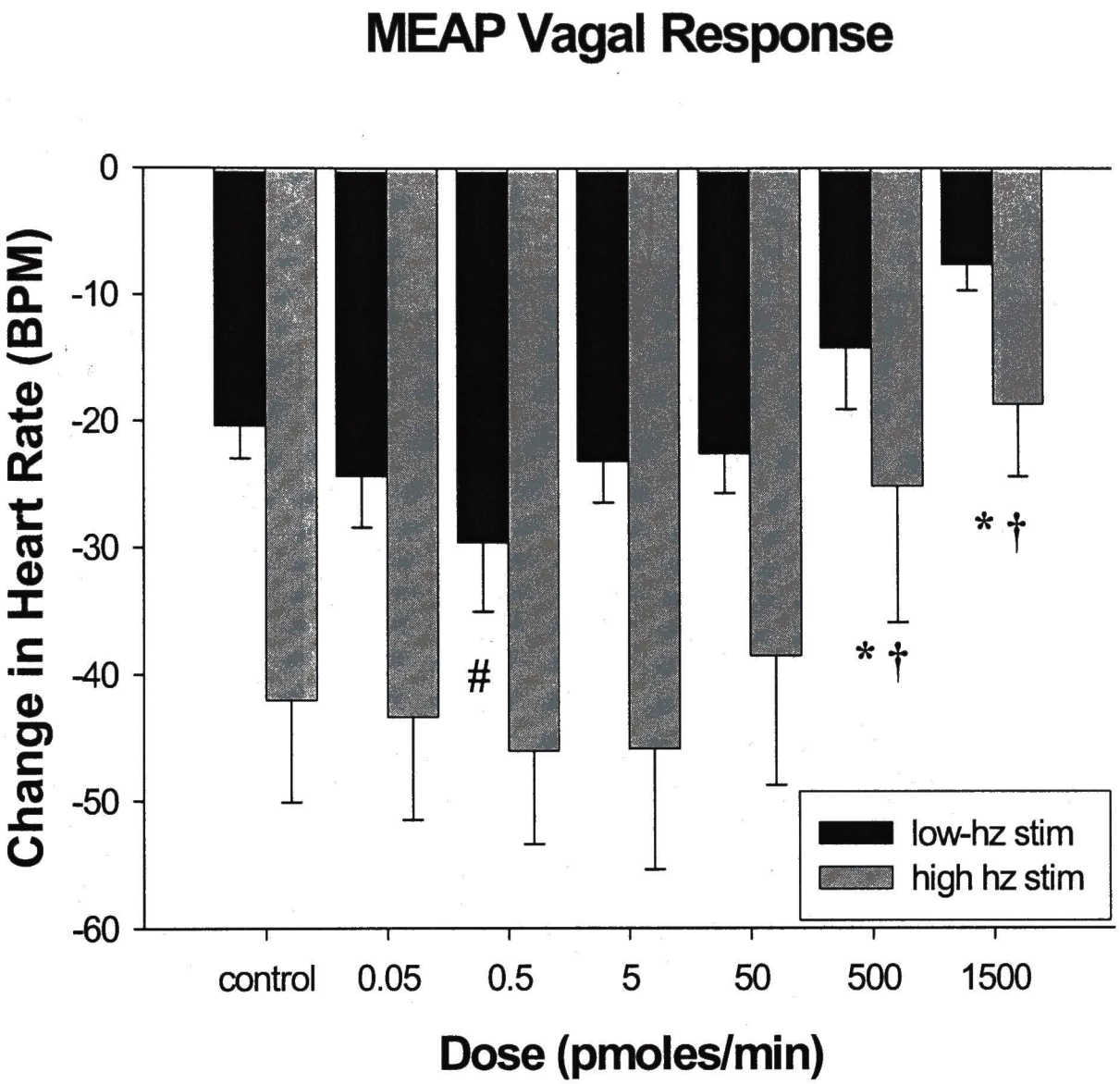
MEAP Vagal Response
Change in Heart Rate (BPM)
0
-10
-20
-30
-40
-50
-60
control
0.05
0.5
5
50
500
1500
Dose (pmoles/min)
low-hz stim
high hz stim
#
* †
* †

Figure 1

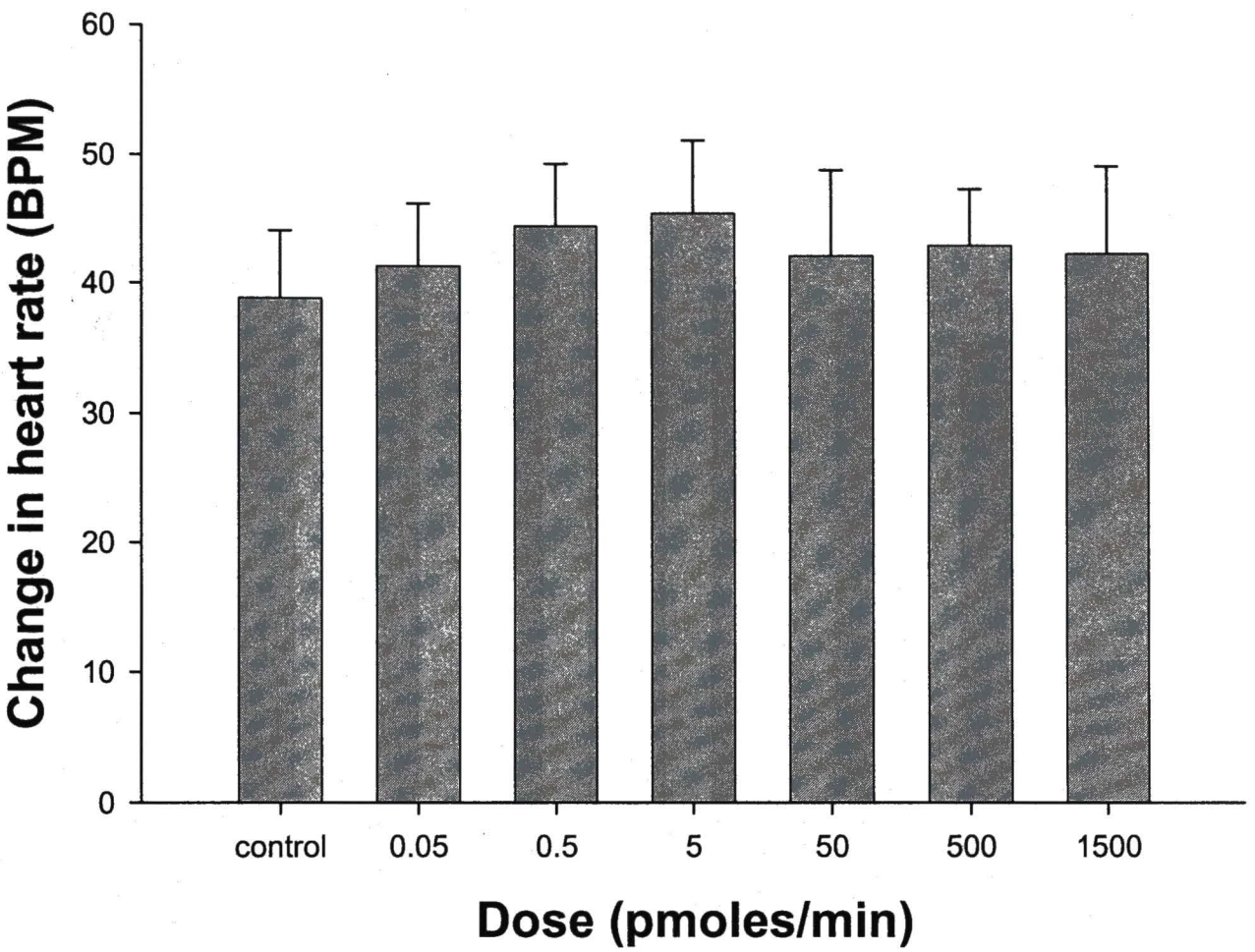
MEAP Sympathetic Response
Change in heart rate (BPM)
60
50
40
30
20
10
0
control
0.05
0.5
5
50
500
1500
Dose (pmoles/min)

Figure 2

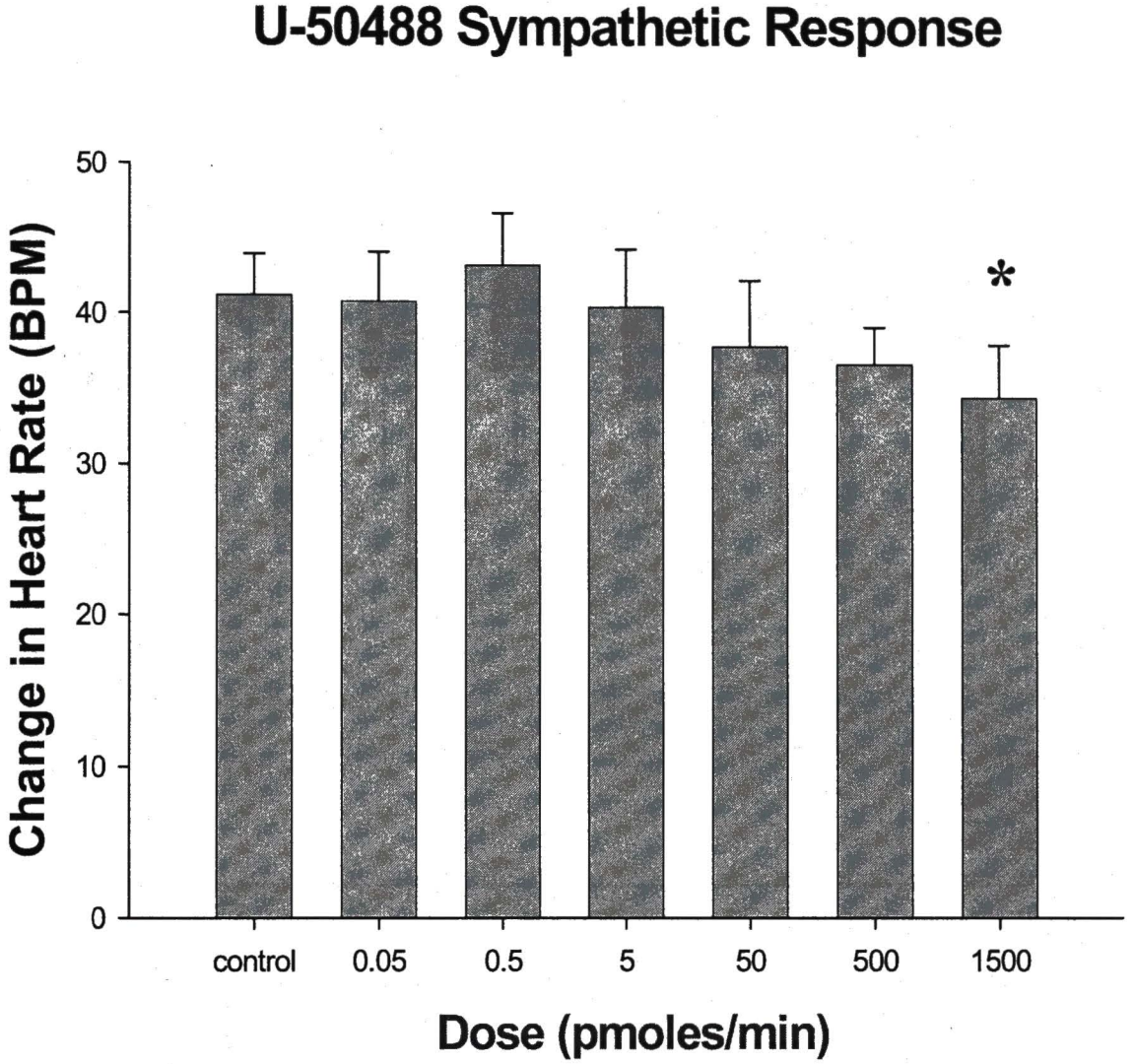
U-50488 Sympathetic Response
Change in Heart Rate (BPM)
50
40
30
20
10
0
*
control
0.05
0.5
5
50
500
1500
Dose (pmoles/min)

Figure 3

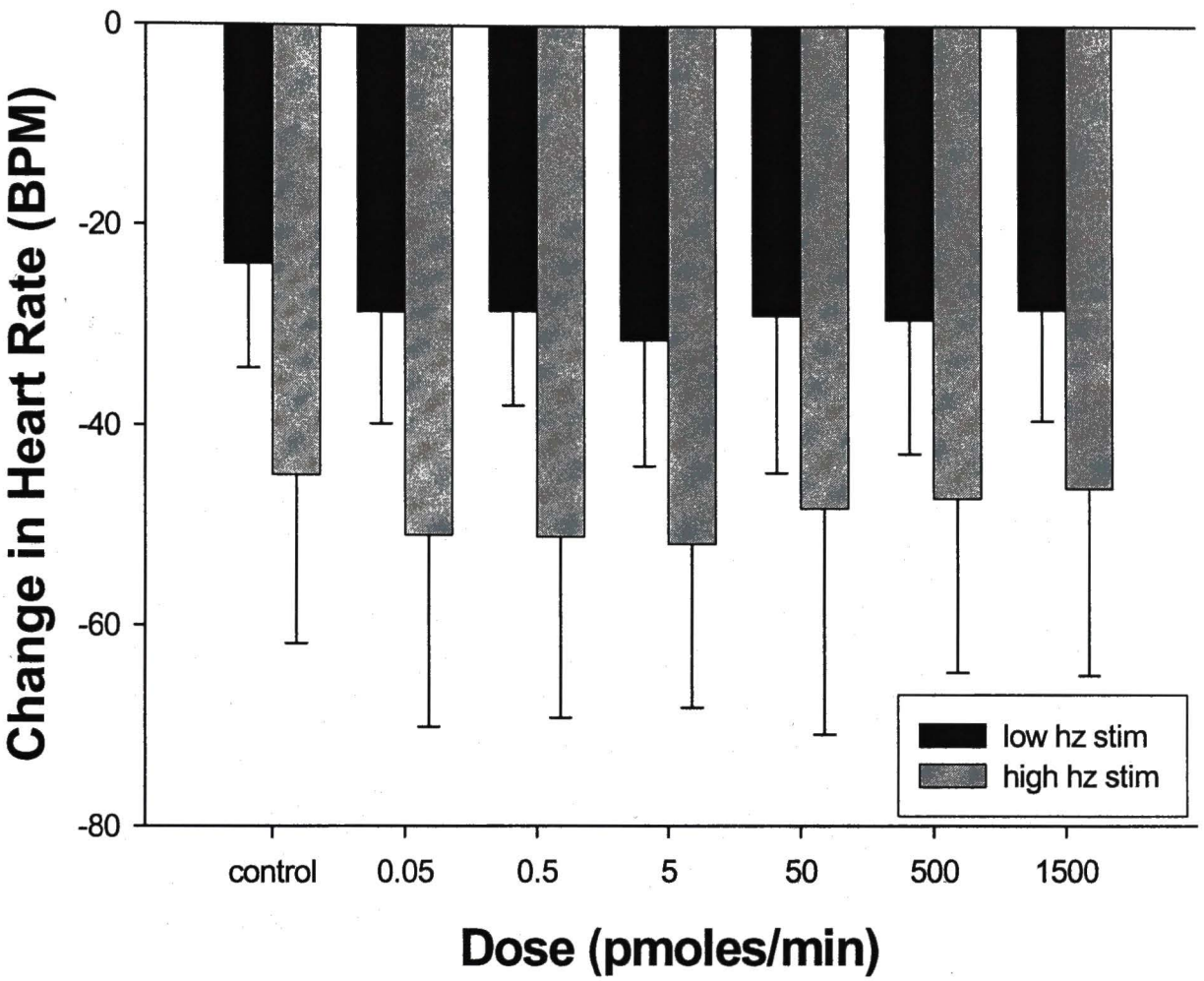
U-50488 Vagal Bradycardia Response
Change in Heart Rate (BPM)
0
-20
-40
-60
-80
control
0.05
0.5
5
50
500
1500
Dose (pmoles/min)
low hz stim
high hz stim

Figure 4

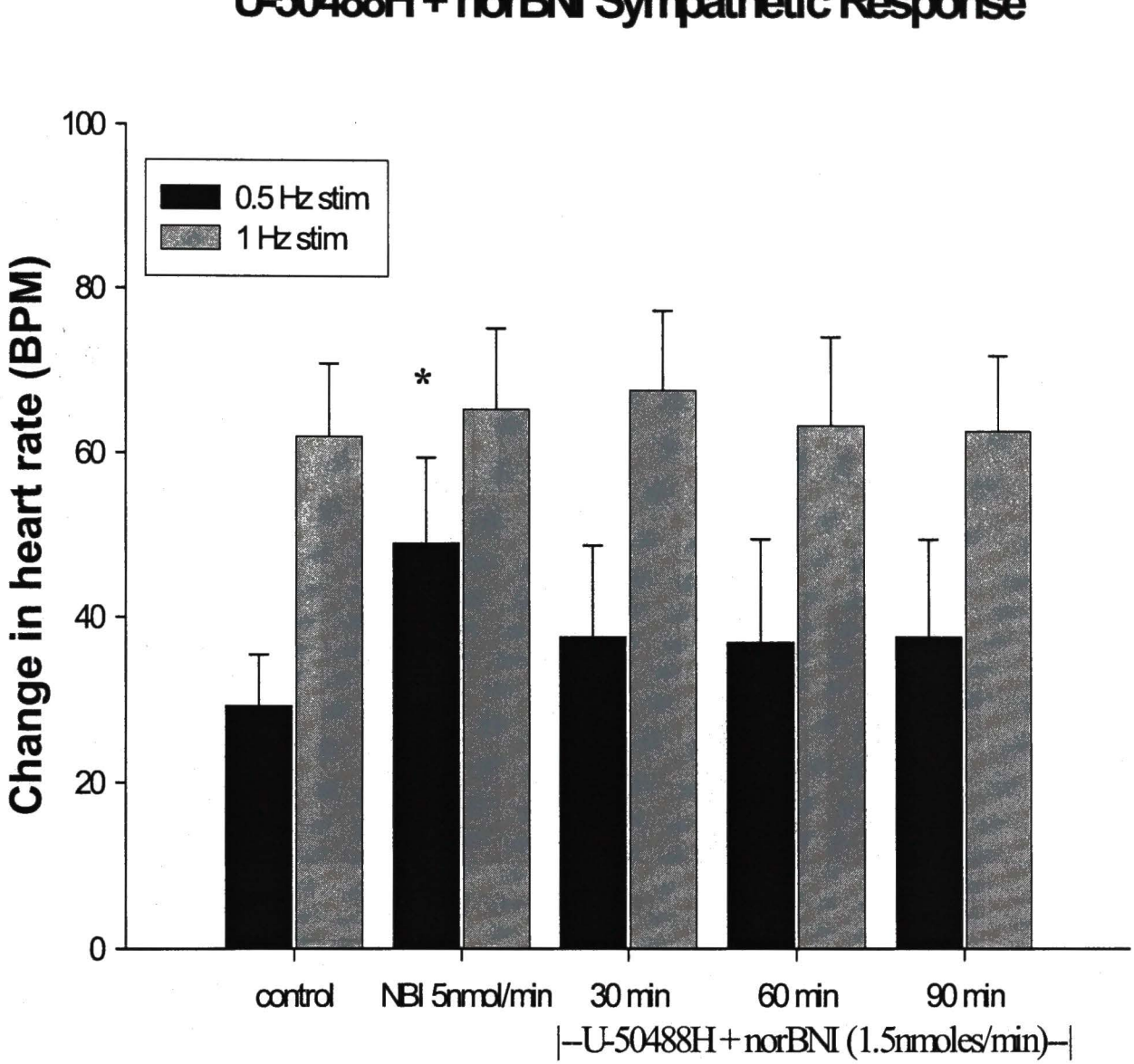
U-50488H + norBNI Sympathetic Response
Change in heart rate (BPM)
100
80
60
40
20
0
0.5 Hz stim
1 Hz stim
*
control
NBI 5nmol/min
30 min
60 min
90 min
|–U-50488H + norBNI (1.5nmoles/min)–|

Figure 5a

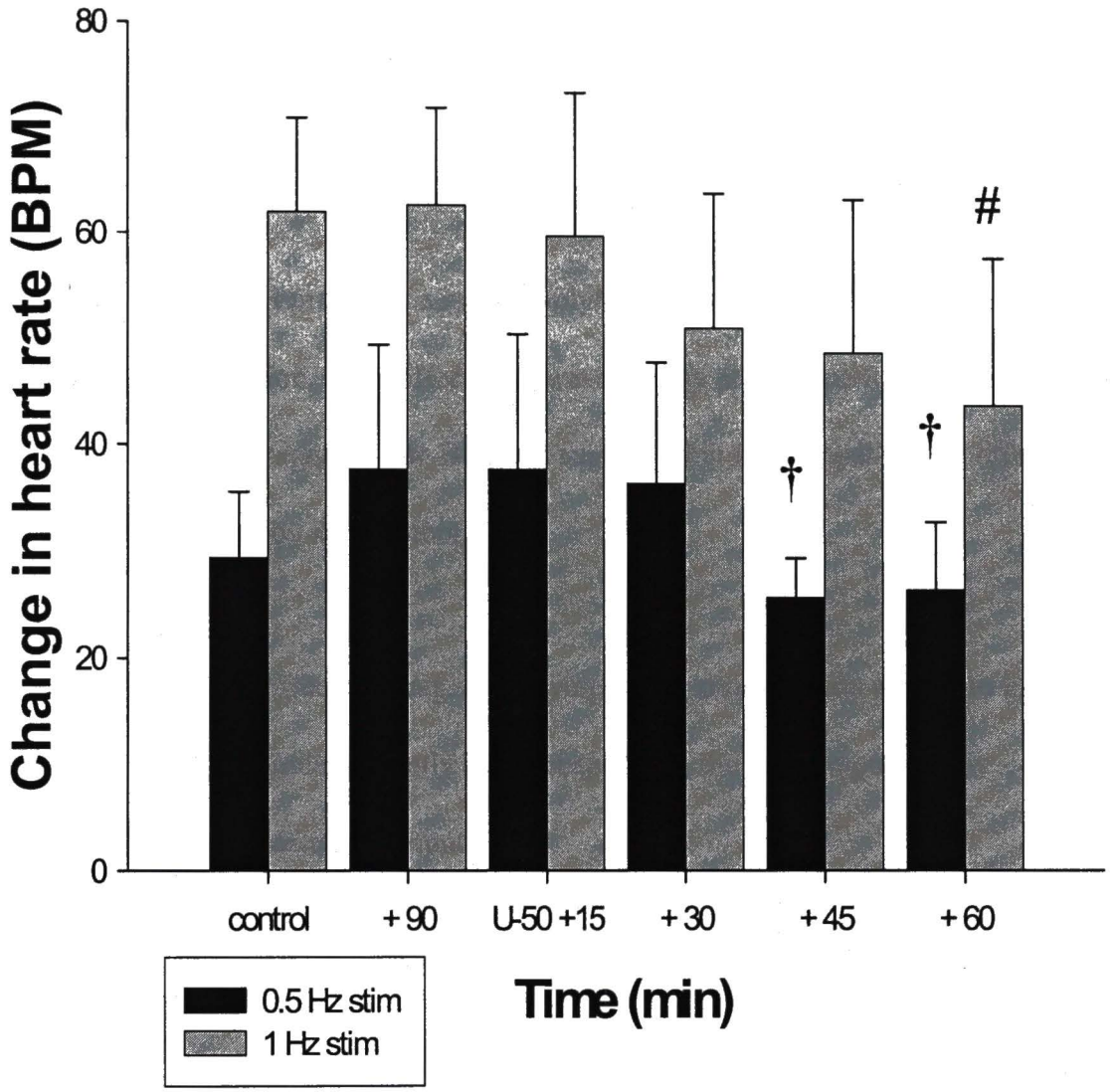
U-50488H Post norBNI Sympathetic Response
Change in heart rate (BPM)
80
60
40
20
0
control
+90
U-50 +15
+30
+45
+60
†
†
#
0.5 Hz stim
1 Hz stim
Time (min)

Figure 5b

CHAPTER III

SUMMARY AND CONCLUSIONS

1. MEAP produced both vagotonic and vagolytic responses without altering sympathetic transmission. Prior studies have determined that these two effects were mediated by δ-opioid receptors.

2. U-50488H produced a sympatholytic effect without altering vagal transmission. This effect was prevented by pretreatment with the selective κ-opioid antagonist, nor-BNI and suggests that the sympatholytic effect was mediated by κ-receptors.

3. Thus the effects on vagal and sympathetic transmission appear to have been mediated independently by δ-receptors on the vagal input and by κ-receptors on the sympathetic input.

4. Finally, the δ-effects appeared to develop and resolve in minutes even when the agonist was applied for an extended interval. In contrast, the κ-effects may, after extended exposure to the agonist, have gradually evolved to a secondary stage that was not easily reversed. Whether this resistance to reversal is characteristic of all κ-agonists or specific to U-50488H remains to be determined.

FUTURE STUDIES

The following studies are proposed to further clarify the cardiovascular effects of the δ- and κ-opioid receptor systems in the sinoatrial node:

1. Use local norepinephrine measurements to test the hypothesis that the sympatholytic effect of κ-receptor stimulation was mediated by inhibition of norepinephrine release.

2. Use immunohistochemistry to determine the anatomical locations of the κ-receptors in the SA node.

3. Use radioimmunoassay and comparative agonist profiles to identify the endogenous agonist responsible for the sympatholytic effect in heart.

4. Use selected biochemical and pharmacological probes to determine the mechanism mediating the sympatholytic effect of κ-receptor stimulation.

5. Finally and perhaps most importantly, to begin studies to determine the importance of these receptors systems and the functional consequences of their activation and/or inhibition.