# Leucine-Enkephalin Interrupts Sympathetically Mediated Tachycardia Prejunctionally in the Canine Sinoatrial Node

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**This study examined the role of leucine-enkephalin (LE) in the sympathetic regulation of the cardiac pacemaker. LE was administered by microdialysis into the interstitium of the canine sinoatrial node during either sympathetic nerve stimulation or norepinephrine infusion. In study one, the right cardiac sympathetic nerves were isolated as they exit the stellate ganglion and were stimulated to produce graded (low, 20–30 bpm; high 40–50 bpm) increases in heart rate (HR). LE (1.5 nmoles/min) was added to the dialysis inflow and the sympathetic stimulations were repeated after 5 and 20 min of LE infusion. After 5 min, LE reduced the tachycardia during sympathetic stimulation at both low (18.2 ± 1.3 bpm to 11.4 ± 1.4 bpm) and high (45 ± 1.5 bpm to 22.8 ± 1.5 bpm) frequency stimulations. The inhibition was maintained during 20 min of continuous LE exposure with no evidence of opioid desensitization. The δ-opioid antagonist, naltrindole (1.1 nmoles/min), restored only 30% of the sympathetic tachycardia. Nodal δ-receptors are vagolytic and vagal stimulations were included in the protocol as positive controls. LE reduced vagal bradycardia by 50% and naltrindole completely restored the vagal bradycardia. In Study 2, additional opioid antagonists were used to determine if alternative opioid receptors might be implicated in the sympatholytic response. Increasing doses of the κ-antagonist, norbinaltorphimine (norBNI), were combined with LE during sympathetic stimulation. NorBNI completely restored the sympathetic tachycardia with an $ED_{50}$ of 0.01 nmoles/min. A single dose of the μ-antagonist, CTAP (1.0 nmoles/min), failed to alter the sympatholytic effect of LE. Study 3 was conducted to determine if the sympatholytic effect was prejunctional or postjunctional in character. Norepinephrine was added to the dialysis inflow at a rate (30–45 pmoles/min) sufficient to produce intermediate increases (35.2 ± 1.8 bpm) in HR. LE was then combined with norepinephrine and responses were recorded at 5-min intervals for 20 min. The tachycardia mediated by added norepinephrine was unaltered by LE or LE plus naltrindole. At the same 5-min intervals, LE reduced vagal bradycardia by more than 50%. This vagolytic effect was again completely reversed by naltrindole. Collectively, these observations support the hypothesis that the local nodal sympatholytic effect of LE was mediated by κ-opioid receptors that reduced the effective interstitial concentration of norepinephrine and not the result of a postjunctional interaction between LE and norepinephrine.** Exp Biol Med 228:898–906, 2003

**Key words:** cardiac pacemaker; enkephalins; opioids; heart rate; sympathetic and parasympathetic nervous systems

The autonomic nervous system (ANS) regulates myocardial contractile activity, coronary blood flow, and heart rate (HR). The sympathetic and parasympathetic innervation of the heart mediate their most obvious actions through the release of norepinephrine and acetylcholine, respectively. The following study was centered on a third, less well-appreciated factor, the endogenous opioids. Several recent reviews have addressed opioid activity in the cardiovascular system (1–3). This study specifically focused on interactions between one opioid, leucine-enkephalin (LE), and the local sympathetic control of the cardiac pacemaker.

As neuromodulators, opioids generally inhibit the release of other neurotransmitters (4–10). Opioids often suppress function by inhibiting excitatory neurotransmitter release, but they can stimulate target organ responses indirectly by inhibiting secretion of neurotransmitters that normally depress function. For example, suppressing the release of acetylcholine would produce an increase in HR or contractile function (5, 9, 14). Practical examples of both excitatory and inhibitory opioid-mediated responses have been reported throughout the cardiovascular system. The direction and character of each response may have depended on the peptide used, the measure evaluated, the route of administration, and the existing autonomic balance at the time (4–19).

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Most inhibitory opioid effects in the circulation have been consistent with interactions at prejunctional targets, regardless of the opioid receptor subtype. Intravenous enkephalin lowered blood pressure (4, 12, 17) in anesthetized animals by disrupting sympathetic ganglionic transmission and thus reducing vasomotor tone (4, 7, 8, 13, 17). Enkephalin, the prototypical δ-agonist, presumably accomplished this by reducing intraganglionic acetylcholine release (7, 8). The intracoronary administration of the κ-selective opioid, dynorphin, reduced sympathetically mediated contractile activity by suppressing the release of norepinephrine (6). Although different opioid receptors were implicated in the two examples (6, 9), the presumed target in each case was the prejunctional nerve terminal and not the heart and vasculature.

Apparent excitatory effects were often observed with enkephalins (5, 9, 13, 22, 23). Intravenous administration of the cardiac enkephalin (20, 21), methionine-enkephalin-arginine-phenylalanine (MEAP), increased HR and inotropic state by interrupting vagal transmission (5, 9–11). Administering a cholinergic agonist circumvented the vagolytic effect of enkephalin and suggested again that the interaction was prejunctional (5). Microdialysis probes placed in the sinoatrial (SA) node were later used to identify the vagal interaction with MEAP as most likely postganglionic and prejunctional (22). Detailed agonist/antagonists profiles have identified the nodal receptor responsible as a δ-opioid receptor (19, 23).

The intensity of the predominant autonomic traffic may in fact determine the quality and intensity of the opioid response observed. Enkephalins produced hypertensive responses when given to conscious animals where vagal activity predominates. In contrast, intravenous enkephalin was hypotensive in the same animals when sympathetic activity was increased after inducing anesthesia (13, 17). The amplitude of the hypotensive response to enkephalin was reversibly increased when sympathetic activity was acutely elevated (12). Consistent again with the hypothesis that the opioids are primarily prejunctional neuromodulators, opioids seldom had any influence in the absence of active nerve traffic.

However, there are convincing reports *in vitro* that support a postjunctional role for cardiac opioids. Two studies, one in isolated cardiomyocytes and the other in isolated heart, reported that enkephalin reduced contractile force when combined with norepinephrine, but not when administered alone (13, 14). Those two postjunctional interactions relied on one specific opioid, LE, and did not address chronotropic activity because both models were paced electrically. The absence of evidence for postjunctional effects *in vivo* may be attributable to differences in the experimental model (*in vitro* versus *in vivo*), the animal model (rat versus dog), in the character of the peptide evaluated (MEAP versus LE), in the rate of degradation, in the dose of opioid, or in the duration of exposure.

The current study was designed to test the hypothesis that in the absence of significant degradation, LE would reduce the sympathetic control of HR through both pre- and postjunctional mechanisms. Microdialysis was used to facilitate access to the cardiac pacemaker and to circumvent degradation en route (22). The discrete anatomical introduction by microdialysis also permits one to sustain interstitial peptide concentrations for an extended duration while minimizing systemic distribution and global hemodynamic compensations.

## Materials and Methods

**Surgical Preparation.** Fifteen mongrel dogs of either gender weighing 15 to 25 kg 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 (32.5 mg/kg), intubated, and mechanically ventilated initially at 225 mls/min/kg with room air. Fluid-filled catheters were then inserted into the right femoral artery and vein and were advanced into the descending aorta and inferior vena cava, respectively. The arterial line was attached to a Statham PD23XL pressure transducer and HR and arterial pressure were measured continuously online (MacLabs, Castle Hill, NSW, Australia). The depth of anesthesia was regularly evaluated and the venous access allowed for administration of supplemental anesthetic as required. The acid-base balance and the blood gases were determined with an Instrumental Laboratories (Lexington, MA) 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 minute volume.

The right and left cervical vagus nerves were isolated through a ventral midline surgical incision. The nerves were double ligated with umbilical tape to eliminate complicating afferent and efferent vagal nerve traffic during the studies. They were then replaced in the cervical compartment for later retrieval. Surgical anesthesia was carefully monitored, and a single dose of succinylcholine (1 mg/kg) was administered intravenously to temporarily reduce involuntary muscle movements during the 10 to 15 min required for electrosurgical incision of the chest and removal of the ribs two through five. A right thoracotomy was performed and the heart was exposed from the right aspect. The pericardium was opened and the pericardial margins were sutured to the body wall to support the myocardium.

**Nodal Microdialysis.** A curved 25-gauge stainless steel spinal needle containing the microdialysis line was inserted into the center of SA node along its long axis (22, 23). The needle was removed and the probe was then positioned so that the dialysis window was completely within the substance of the SA node. .The inflow line was then attached to a microinfusion pump and perfusion of the probe

with Krebs ringer was initiated at 5 μl/min as described by Van Wylen *et al.* (24). The SA node can be identified in the canine heart as a faint pale area located at the junction of the superior vena cava and the right atrium. The functional location of the probe was verified in each animal by the observation of a brisk tachycardia after the brief introduction of norepinephrine (1 ng/μl) into the probe.

In two groups of the animals, the cardiac nerves distal to the right stellate ganglion (right ansa subclavia) were isolated and briefly tested to ascertain the frequencies needed to produce consistent lower (0.5–1.25 Hz, 20–30 bpm) and higher (0.75–1.50 Hz, 40–50 bpm) increases in HR. In the third group, increasing doses of norepinephrine were introduced into the dialysis line to determine the dose (30–45 pmoles/min) necessary to produce an intermediate, submaximal (20–40 bpm) increase in HR. Once the appropriate dose was determined, the norepinephrine was discontinued. In both protocols, the system was then perfused with Krebs ringer at 5 μl/min for 1 hr to permit the tissue to re-equilibrate before beginning either experimental protocol. Previous studies reported a similar equilibration period was sufficient to restore baseline interstitial conditions (24).

**Study 1: LE and Sympathetic Stimulation.** After 1 hr of perfusion with vehicle, the right side sympathetic nerves (ansa subclavia) were stimulated for 30 sec at a low frequency (0.50–1.25 Hz) predetermined earlier in each animal to produce a 20 to 30 bpm increase in HR. The system was then allowed 2 min to recover and then the nerves were stimulated for 30 sec again at a higher frequency (0.75–1.5 Hz) previously determined in each animal to produce a 40 to 50 bpm increase in HR. Fifteen minutes were allowed for re-equilibration from the control stimulation, and then LE was added to the dialysis line and perfused at 1.5 nmoles/min. The sequence of sympathetic stimulations (low or high) was then repeated after 5 min of nodal perfusion with LE. The dose was selected based on prior dose responses with related peptides in the same model system (22–23). Prior observations *in vitro* (18) indicated that interactions between LE and norepinephrine required minutes to evolve. To assess for a slowly developing effect, the LE infusion was continued and the two sympathetic stimulations were conducted once again 15 min later at 20 min. The opiate antagonist, naltrindole (1.1 nmoles/min), was then added to the perfusate with LE and the stimulations were repeated 5 min later at 25 min. Because prior studies clearly implicated enkephalins as moderating vagal function (5, 22, 23), vagally mediated bradycardia was evaluated in each experiment as a positive control. After each set of sympathetic stimulations, the right vagus nerve was stimulated at a supramaximal voltage (15–20 V, 5 msec) for 15 sec followed by 1 min and 45 sec for recovery. In each instance, the vagal stimulation was first conducted at 1 Hz and was then repeated 2 min later at 3 Hz. After completing the treatments, the dialysis perfusate was switched back to vehicle for 10 min, and as an internal control, the nerve function was re-evaluated to demonstrate complete recovery of nerve function. In addition to the internal control, repeated sympathetic stimulation "time controls" were conducted at the end of the protocol in two of the Study 3 animals to verify that multiple stimulations alone did not erode or enhance the subsequent HR response. After completing the Study 3 protocol below, recovery from the treatments was verified by the restoration of normal vagal function. The probe was then perfused with vehicle for 20 min and the HR response to sympathetic stimulation was tested at 5 and 20 min to evaluate the effect of repeated stimulations alone.

**Study 2: LE, Sympathetic Stimulation, and Norbinaltorphimine (NorBNI).** The protocol for this study was similar to Study 1, however, fewer stimulations were conducted during each treatment to minimize the total number of stimulations. All stimulations were conducted after 5 min of treatment and at one sympathetic stimulation frequency (0.75–1.50 Hz). The frequency was selected empirically in each animal to produce a 35 to 50 bpm increase in HR. After each treatment or dose, the dialysis perfusate was restored to saline and the system was permitted to recover for 30 min before the initiating the next treatment. The treatments were applied in sequence as follows: vehicle, LE alone (1.5 nmoles/min), LE (1.5 nmoles/min) combined with step increases in the κ-antagonist, Nor BNI (0.01, 0.03, 0.1, 0.3, and 1 nmoles/min), norBNI alone (1.0 nmoles/min), and LE alone (1.5 nmoles/min). In three animals, the reintroduction of LE was followed by two additional treatments that included LE (1.5 nmoles/min) combined with the μ-antagonist, CTAP (1.0 nmoles/min), and CTAP alone (1.0 nmoles/min).

**Study 3: LE and NE Infusion.** After the 1 hr of vehicle perfusion, norepinephrine was added to the dialysis perfusate and was perfused at a dose rate determined earlier in the protocol as sufficient to increase HR 20 to 40 bpm. The concentration of norepinephrine required to evoke the desired increase in HR ranged from 30 to 45 pmoles/min. The norepinephrine was then discontinued and the tachycardia was allowed to subside during perfusion with vehicle. Recovery of the pretreatment HR usually required 5 to 10 min. Once the baseline HR was re-established, LE (1.5 nmoles/min) and norepinephrine were combined and the node was perfused with the combination. The HR was then recorded at 5-min intervals during the following 20 min. After 20 min, the opioid antagonist, naltrindole (1.1 nmoles/min), was added to the other two agents in the perfusate and the HR was re-evaluated 5 min later. All agents were then washed out and the function was again allowed to return to baseline. Again, vagal function served as a positive control and the response to right vagal stimulation was evaluated at 3 Hz, before, during each treatment combination, and again after washout.

**Materials.** LE (Phoenix Pharmaceuticals, Mountain View, CA), norBNI (Tocris Cookson, Ellisville, MO), CTAP, and naltrindole (Sigma Chemical, St. Louis, MO) were obtained from commercial sources. All peptide calculations were adjusted for net peptide content. The microdi-

**Table I.** Resting HR and MAP Before Sympathetic Stimulation or NE Administration

| Cardiovascular indices | | | | |
|---|---|---|---|---|
| **Study one: sympathetic stimulation** | | | | |
| | Pre-LE | LE-5 | LE-20 | Post-LE |
| HR (bpm) | 116 ± 1.9 | 116 ± 2.0 | 113 ± 1.7 | 104 ± 2.2 |
| MAP (mmHg) | 95 ± 0.9 | 95 ± 1.2 | 93 ± 1.0 | 91 ± 2.5 |
| **Study two: sympathetic stimulation + κ-antagonist** | | | | |
| | Pre-LE | LE | LE + norBNI | norBNI |
| HR (bpm) | 126 ± 4.3 | 129 ± 4.7 | 124 ± 5.3 | 126 ± 5.4 |
| MAP (mmHg) | 114 ± 5.4 | 123 ± 5.0 | 124 ± 6.5 | 126 ± 7.7 |
| **Study three: NE infusion** | | | | |
| | Pre-NE | PreNE + LE | Post-NE | |
| HR (bpm) | 132 ± 2.4 | 132 ± 2.4 | 127 ± 1.9 | |
| MAP (mmHg) | 101 ± 1.4 | 102 ± 1.2 | 100 ± 1.4 | |

The values are means and SEM. There was no significant effect of LE or the κ-antagonist, norBNI on the resting HR or MAP. All values were recorded after 5 min of treatment except LE-20, which was recorded after 20 min of treatment.

alysis probes were fabricated in our laboratory as described by Van Wylen *et al.* (24).

The microdialysis probes for Studies 1 and 3 were constructed of a single 1-cm length of dialysis fiber from a Clirans 10 (Asahi Medical, Tokyo, Japan) artificial kidney (24). The dialysis tubing (300-μm ID; 305-μm OD) had a molecular weight exclusion of 5,000 kD. The inflow and outflow lines were constructed of hollow 170-μm OD silica tubing (SGE, Austin, TX) glued into the dialysis fiber. The dialysis fiber was no longer available when probes for Study 2 were fabricated. A smaller diameter dialysis fiber (220-μm OD, 200-μm ID) from a Clirans TAF-08 (Asahi Medical) artificial kidney with a larger molecular weight exclusion, 36,000 kD, was substituted.

**Data Analysis.** Analysis of variance for repeated measures with a block design was used for most comparisons. Subsequent *post hoc* comparisons were made with either Tukey's or Dunnett's tests. Differences were considered significant at $P < 0.05$.

## Results

The initial cardiovascular indices for all animals are listed in Table I. There is no obvious explanation except normal variation for the differences in initial values between the three groups of randomly selected animals. Also, there were no significant treatment effects on resting function thereafter.

**Study I: LE and Sympathetic Stimulation.** The sympathetic nerves were isolated as they exited the right stellate ganglion and they were stimulated for 30 sec at the low frequency (0.5–1.25 Hz). They were then allowed to recover and were then stimulated again at the higher frequency (0.75–1.5 Hz). The lower and higher frequency stimulations produced, respectively, mean increases of 18.2 ± 1.3 bpm and 45 ± 1.4 bpm (Fig. 1). During LE infusion, the low frequency increase in HR was reduced by one-third to 11.4 ± 1.4 bpm after 5 min. No slowly developing enkephalin effect was apparent because an additional 15 min of exposure to enkephalin produced a nearly identical reduction in tachycardia to 10 ± 1.0 bpm. At the higher frequency, the enkephalin-mediated inhibition exceeded 50% and, again, the reduced tachycardia was nearly identical at 5 and 20 min (to 22.8 ± 1.5 bpm vs 23 ± 1.7 bpm) of exposure to enkephalin. The introduction of the opiate antagonist, naltrindole, into the perfusate partially restored the sympathetically mediated tachycardia (Fig. 1). The nerve-

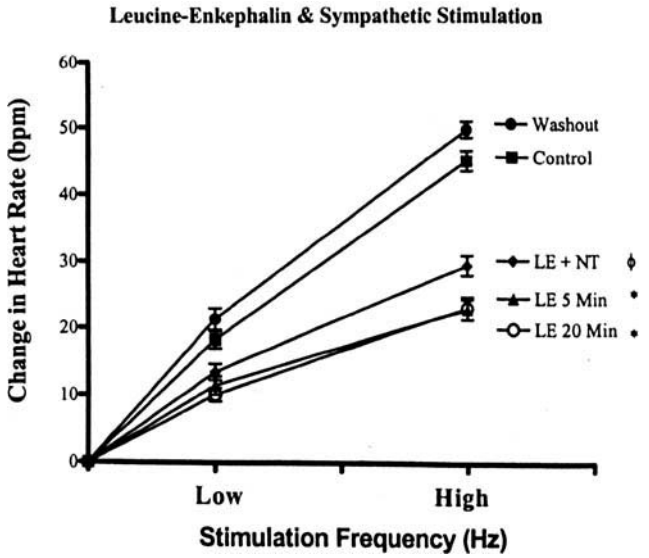


**Figure 1.** Changes in HR mediated by sympathetic stimulation are illustrated during nodal delivery by microdialysis of vehicle (■),LE at 5 min (▲), LE at 20 min (○), LE + naltrindole (LE + NT) (♦), and after washout of node with vehicle (●). Values are means and SEs from five subjects. Sympathetic stimulation during LE infusion was significant reduced compared with control, $P < 0.05$ (*). Sympathetic stimulation plus LE plus naltrindole was significantly different from both vehicle and LE (ϕ).

stimulated increase in HR was greater than that observed with LE alone, but the partial increase with naltrindole was still statistically reduced compared with the preopioid controls (45 ± 1.4 bpm vs 29.4 ± 1.5 bpm vs 23 ± 1.7 bpm). Treatments were discontinued and the system was perfused again with vehicle. Sympathetic stimulations once again yielded increases in HR not significantly different from the original control responses. The restoration of control responses confirmed that the decline in response during LE was not a repeated stimulation effect. Sequential sympathetic stimulations were further evaluated at 5 and 15 min of vehicle infusion in two additional animals. The increase in HR in these time controls was similar (low Hz: 17 ± 2.5; high Hz: 43.75 ± 1.7) to that observed in the larger group and the increase was identical at 5 and 20 min with no apparent decline due to repeated stimulation.

The right cervical vagus nerve was stimulated at 1 and 3 Hz before, during, and after LE infusion. HR, as expected, quickly declined in a frequency-dependent fashion. The average declines in HR at 1 and 3 Hz were 11.8 ± 1.4 bpm and 45.6 ± 5.4 bpm, respectively. During enkephalin infusion, vagal responses were attenuated by 50% and, like the sympathetic responses described above, the interruption of vagal function was maintained for the duration of the 20-min perfusion (Fig. 2). The average decline in HR was reduced by enkephalin to 5 to 6 bpm at 1 Hz and 16 to 25 bpm at 3 Hz. Unlike the sympathetic responses, naltrindole completely restored vagal bradycardia to the pretreatment control response. When naltrindole and LE were combined, the resulting vagal bradycardia during low and high frequency (1 Hz: 10 ± 0.4 bpm and 3 Hz: 44 ± 5.1 bpm) stimulation was not different from control. Finally, vagal responses were evaluated again after the perfusate was replaced with vehicle and the enkephalin and naltrindole were allowed time to wash out. The reductions in HR during these last vagal stimulations were once again very similar to the control responses (11.3 ± 0.6 bpm and 55 ± 3.2 bpm).

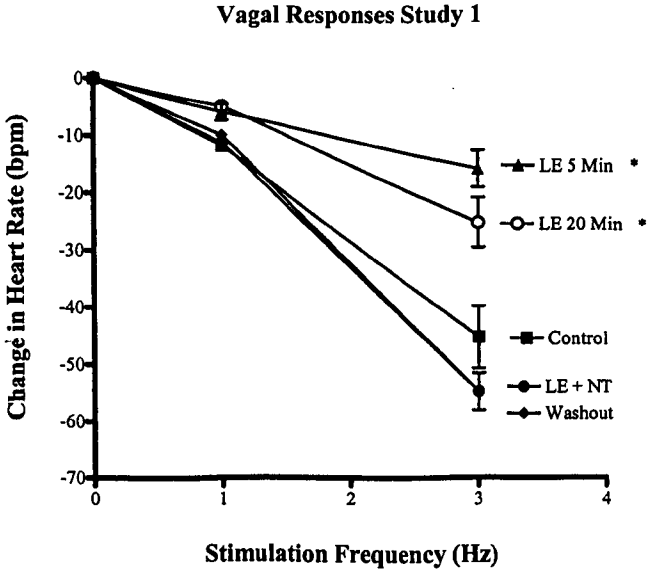


**Figure 2.** Changes in HR mediated by vagal stimulation are illustrated during the nodal delivery by microdialysis of vehicle (■), during LE at 5 min (▲), during LE at 20 min (○), during LE + naltrindole (NT) (●) and after washout of node with vehicle (◆). Values are means and SEs from five subjects. Vagal stimulation during LE infusion was significantly different from control (*) ($P < 0.01$).

**Study 2: LE, Sympathetic Stimulation, and NorBNI.** The weak reversal by naltrindole observed in Study 1 suggested that the sympatholytic effect of LE might involve a non-δ-opioid receptor. In this regard, Figure 3 illustrates the effect of combining LE with increasing doses of the κ-antagonist, norBNI. The sympathetic stimulation produced a reproducible 40 to 50 bpm increase in HR. LE reduced the tachycardia by more than 50% (42 ± 3 bpm vs 17 ± 3 bpm). NorBNI completely reversed the sympatholytic effect of LE with maximal effect at 0.03 nmoles/min and an estimated $ID_{50}$ of 0.01 nmoles/min. After washing out the last LE-norBNI combination, norBNI alone was tested and was not different from control. LE was then reintroduced to demonstrate that the reversal by norBNI was due to receptor antagonism and not due to desensitization to repeated exposure to LE. After re-establishing the LE sympatholytic effect, LE was combined with the μ-antagonist, CTAP ($n = 3$), at a molar dose rate equivalent to the maximum dose of norBNI. In contrast, CTAP was unable to alter

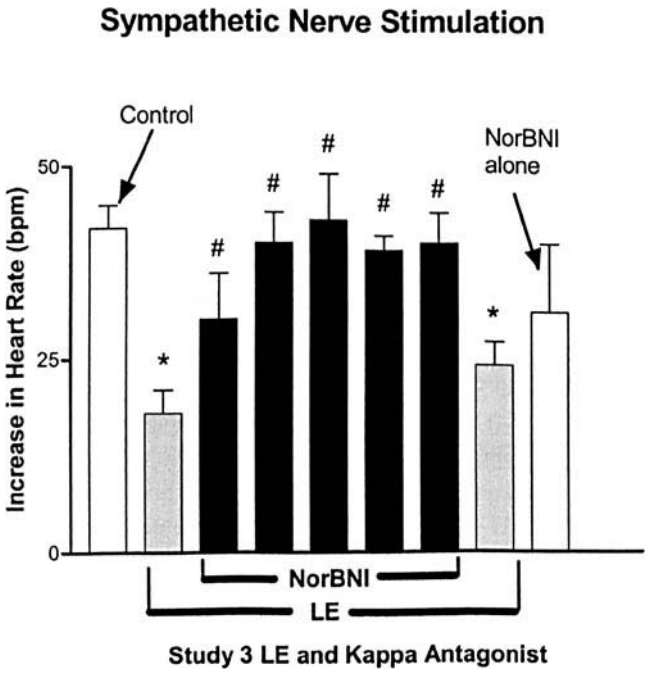


**Figure 3.** Changes in HR mediated by sympathetic stimulation are illustrated during nodal delivery of vehicle (control), LE (gray bars), LE + norBNI (black bars), or norBNI alone by microdialysis. A constant dose of LE (1.5 nmoles/min) was combined with step increases in the κ-antagonist, norBNI (0.01, 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 SEs from five subjects. Sympathetic stimulation during LE infusion was significantly reduced compared with control, $P < 0.05$ (*). NorBNI reversed the LE-mediated sympatholytic effect with an apparent ID50 of 0.01 nmoles/min. The symbol (#) indicates norBNI + LE combinations that were significantly different from LE, $P < 0.05$.

the sympatholytic effect of LE (19 ± 1 bpm vs 19 ± 2 bpm), and both LE and LE + CTAP were different from CTAP alone (38 ± 7 bpm, $P < 0.05$). Thus, κ-opioid and not μ- or δ-opioid receptors are the most likely candidates for mediating the sympatholytic response. More complete agonist antagonist profiles would be necessary to verify that thesis.

**Study 3: LE and NE Infusion.** In Study 3, norepinephrine was infused into the SA node by microdialysis to evaluate a potential postjunctional opioid interaction. Norepinephrine was titrated in each animal and was then perfused at a concentration sufficient to produce an increased HR (35.2 ± 1.8) similar to those observed during sympathetic stimulation in Study 1. After 5 min of equilibration, the HR was recorded and the norepinephrine in the perfusate was discontinued. The HR was then allowed to return to basal values. Once basal HR was restored, the selected dose of norepinephrine was combined with LE and the node was perfused for 20 min. The HR was recorded at 5-min intervals. The response to the norepinephrine/enkephalin combination was very consistent throughout the 20-min perfusion and was indistinguishable from the prior response to norepinephrine alone (Fig. 3). The subsequent addition of naltrindole to the mixture likewise had no effect on the response to norepinephrine.

In the absence of a sympathetic response, vagal function was evaluated to verify that each of the constituents had arrived in the nodal interstitium. The right cervical vagus nerve was stimulated (3 Hz) under each condition. Vagal stimulation reduced HR by 51.25 ± 4.8 bpm in the absence of catecholamine (Fig. 4). The HR was significantly higher during norepinephrine, and, as expected from principle of accentuated antagonism, the vagal effect on HR was much greater. The vagal stimulation now reduced HR an average of 96 ± 10.3 bpm. Once the norepinephrine was washed out, the vagal response returned to control values, 48.8 ± 4.2 bpm. After 5 min of perfusion with the norepinephrine/enkephalin, the vagal stimulation yielded a much less robust bradycardia (59.0 ± 13.2 bpm vs 96 ± 10.3 bpm). In addition, when naltrindole was added to the norepinephrine/enkephalin mixture, the full vagal response (87 ± 7.1 bpm) was restored (Fig. 4). Once again, when all agents were washed out, vagal responsiveness was returned to control (49.8 ± 4.4 bpm).

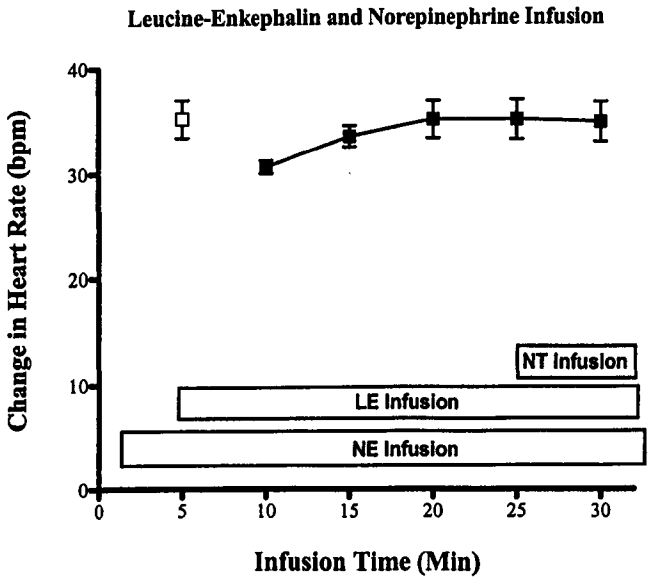


**Figure 4.** Changes in HR are illustrated during the nodal delivery of NE, NE + LE, and NE + LE + naltrindole (NT) by microdialysis. No treatment effects were observed for LE or NT.

## Discussion

Opiates are generally recognized as neuromodulators that exert their effects via prejunctional inhibition of neurotransmitter release. However, several investigators have reported significant postjunctional interactions in the cardiovascular system (15, 17). The two prominent reports of postjunctional interactions in the rat heart both used LE. In both cases, the depressant effect of enkephalin was observed *in vitro* and appeared to be dependent on an interaction with added catecholamines. The low concentrations of agonist and antagonists in both of these studies provided convincing support for the existence of a postjunctional opioid interaction. Similar postjunctional effects were not observed in the dog heart *in vivo* (6), perhaps due to differences in the peptide used, the duration of exposure, the degradation of the peptide en route to the target, the animal model, or the route of administration. In the current study, microdialysis was used to circumvent LE metabolism en route to the SA node to determine whether LE exerts a negative chronotropic effect, whether LE acts at pre- or postjunctional sites, and whether its effects are slow to develop and therefore require an extended duration of exposure.

Efferent cardiac sympathetic nerve stimulation produced a brisk frequency-dependent tachycardia that was reduced when LE was introduced into the nodal interstitium. LE had no effect on the spontaneous HR. These observations suggested that enkephalin depressed either the prejunctional release of norepinephrine or the postjunctional effect of norepinephrine once released. The absence of a LE effect on the resting HR, although suggestive of a prejunctional site, does not rule out a postjunctional interaction. The expression of the sympatholytic interaction might require a threshold level of postjunctional adrenergic stimulation. In fact, the studies that implicated a postjunctional interaction for LE also reported that LE had little effect without added norepinephrine (15, 17). The earlier failure of intracoronary LE to reduce sympathetic tachycardia in the dog may have resulted from rapid degradation of the peptide in route to the target (9).

The failure of the δ-selective antagonist, naltrindole, to restore sympathetic control during enkephalin administration suggested that the sympatholytic effect was either nonopioid or was mediated by another opioid receptor subtype. Vagal function was evaluated as a positive control to assure that an effective dose of naltindole was achieved locally

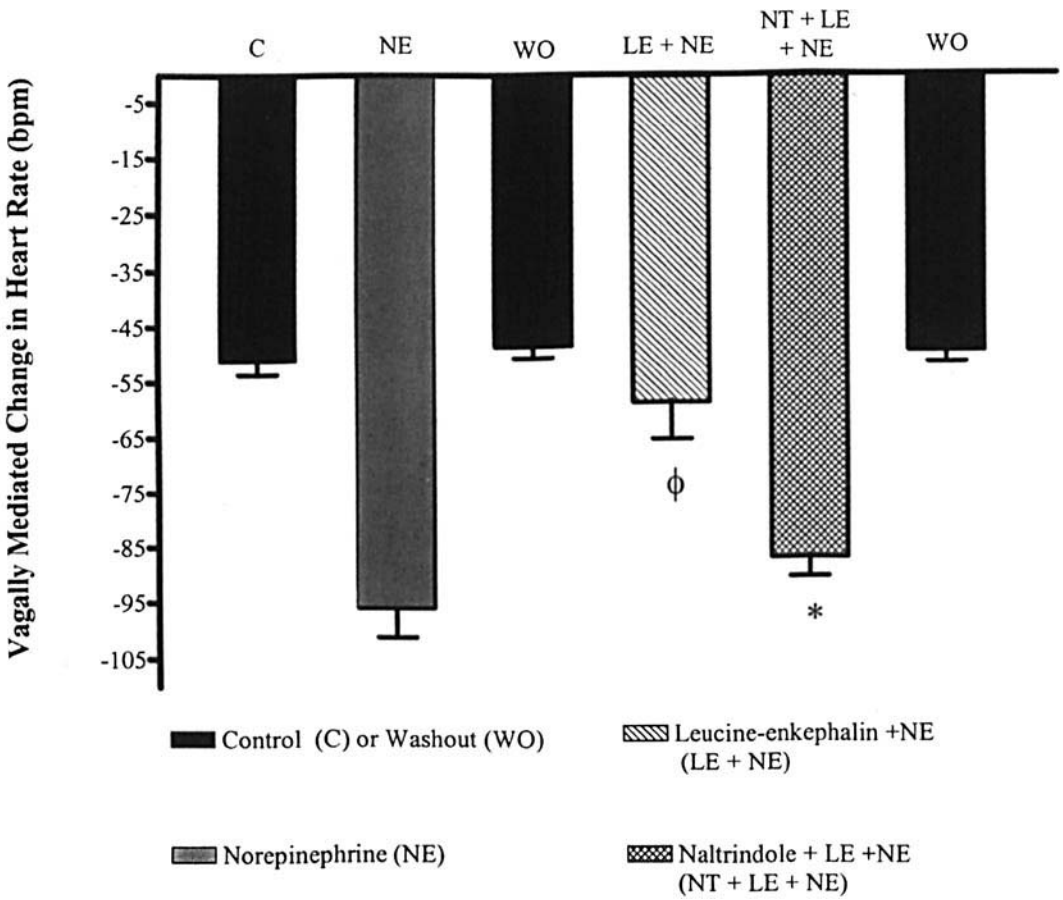


**Figure 5.** Changes in HR mediated by vagal stimulation are illustrated during the nodal delivery by microdialysis of vehicle (C), during NE, during washout of the node with vehicle (WO), during LE + NE, and during NT + LE + NE by microdialysis. Values are means and SEs from five subjects. Vagal stimulation during NE infusion was significantly different from vagal stimulation during NE + LE (ϕ) ($P < 0.01$). NT+LE+NE infusion was significantly different from NE + LE (*) ($P < 0.05$).

within the node. The utility of evaluating vagal function was based on prior observations that assorted enkephalins were vagolytic (5, 9, 11, 14). Although the natural opioid peptides are not entirely selective for one opioid-receptor subtype, the vagolytic effect of intranodal enkephalin had already been carefully classified as a δ-receptor interaction (23). Nodal LE produced a vagolytic effect similar to that observed with other enkephalins (5, 9, 11, 14, 22, 23), and the effect was completely reversed when LE and naltrindole were combined. If μ- or κ-receptors were involved, the partial reversal of the sympatholytic effect by naltrindole would be consistent with the weak antagonist activity of naltrindole at μ- and κ-receptors. The reversal by the κ-antagonist, norBNI, and failure of the μ-antagonist, CTAP, suggested that the sympatholytic receptor was most likely a κ-receptor. Verification of the κ-receptor hypothesis will require more extensive agonist/antagonist profiles. However, participation by prejunctionally located κ-receptors would be consistent with the report that the κ-agonist, dynorphin, inhibited coronary norepinephrine overflow during sympathetic stimulation (6).

Study 3 was designed to determine whether the observed sympatholytic effect in Study 1 had a postjunctional component. Norepinephrine introduced into the nodal interstitium by microdialysis generated a sustained reproducible tachycardia of similar intensity to that obtained with nerve stimulation. When LE was combined with norepinephrine, the norepinephrine-induced tachycardia was unaltered. This strongly suggested that the sympatholytic effect observed during nerve stimulation had been mediated prejunctionally. The absence of an enkephalin effect during norepinephrine administration raised concern that administered norepinephrine might have prevented enkephalin from reaching the pacemaker. However, the coincident vagolytic effect of LE suggested that sufficient LE had reached the nodal targets and interference from norepinephrine was unlikely. The combined vagolytic and absent sympatholytic effects of LE during norepinephrine administration reinforce the inference that the observed sympatholytic effects of LE during sympathetic nerve stimulation were prejunctional.

Vagally mediated bradycardia was significantly greater (accentuated antagonism) during the infusion of norepi-

nephrine. The efficacy of enkephalin as a vagolytic agent was also equal to or greater than that observed in the absence of norepinephrine. This was somewhat surprising because vagal prejunctional α-2-adrenergic and δ-opioid receptors are proposed to exert their inhibitory actions via a closely related Gi/Go-mediated inhibition of adenylyl cyclase. The additional inhibition of vagal function by enkephalin in the presence of continuous exposure to the adrenergic agonist suggested that the two inhibitory (opioid and adrenergic) mechanisms are somehow segregated and perhaps complimentary. Extensive dose-response relationships would likewise be needed to verify that suggestion.

Many opioid systems are characterized by the rapid desensitization of the response during continuous exposure to the opioid. Interestingly, both the sympatholytic and vagolytic responses to intranodal enkephalin were maintained unchanged throughout the 20-min exposure. The rapid recovery of normal neuroendocrine control soon after washout was consistent with the aggressive degradation of enkephalin by ever-present aminopeptidases (25). The rapid recovery and the apparent prejunctional character of the interactions reinforce the hypothesis that cardiac enkephalins are primarily paracrine and neuromodulatory.

Normal cardiac function requires an organized electromechanical coupling that is normally modulated by a delicate balance of sympathetic and parasympathetic influences. An orderly electrical rhythm would be challenging to maintain during intense sympathetic or parasympathetic stimulation. LE may act as a governor to prevent overstimulation of the heart by either limb of the autonomic nervous system. Opiates have been implicated in myocardial ischemia and ischemic preconditioning (3, 26–28). The release of enkephalin in the area at risk could reduce sympathetic activity locally provided that enkephalin behaves similarly in the non-nodal myocardium. Lowered local norepinephrine concentrations would reduce oxygen demand in the area at risk while simultaneously allowing normal adrenergic transmission in areas where the coronary blood supply was adequate. Together, these two activities could help preserve the compromised myocardium and at the same time facilitate global myocardial contractile function and a return to a normal cardiac output. The relative importance of opioids and other potential paracrine mediators like adenosine, ATP, and nitric oxide, remains to be determined.

In summary, intranodal LE reduced sympathetic control of HR through interaction with an apparent prejunctional κ-opioid receptor. Nodal LE simultaneously interrupted local vagal transmission through interaction with an apparent δ-opioid receptor. The vagolytic effect was comparable with that observed previously for methionine-enkephalin and methionine-enkephalin-arginine-phenylalanine (5, 9, 11, 14, 22, 23). Explaining the differential effect of the same peptide on opposing autonomic efferents is problematic unless one invokes some form of compartmentalization within the node. However, because LE is an unlikely κ-agonist, physiologically, the sympatholytic activity may be mediated by another agonist with greater κ-selectivity (e.g., dynorphin). LE may only be sympatholytic only when present in excess. Finally, the absence of evidence for a postjunctional response cannot be attributed to an insufficient duration of exposure or degradation en route to the target. This differs from the postjunctional interaction between LE and norepinephrine reported for contractile activity in isolated heart, and isolated cardiomyocytes from rats. The apparent contradiction may still be attributed to differences in the model (*in vivo* versus *in vitro*) or in the species (dog versus rat). Postjunctional effects observed *in vitro* (15, 17) may also be more subtle and thus more difficult to demonstrate *in vivo* in the presence of predominant, prejunctional, neuromodulatory influences.


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