Left vagal stimulation induces dynorphin release and suppresses substance P release from the rat thoracic spinal cord during cardiac ischemia

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ABSTRACT

Electro-stimulatory forms of therapy can reduce angina that arises from activation of cardiac nociceptive afferent fibers during transient ischemia. This study sought to determine the effects of electrical stimulation of left thoracic vagal afferents (C8-T1 level) on the release of putative nociceptive (substance P; SP) and analgesic (dynorphin; DYN) peptides in the dorsal horn at the T4 spinal level during coronary artery occlusion (CoAO) in urethane anesthetized Sprague-Dawley rats. Release of DYN and SP was measured using antibody-coated microprobes. While DYN and SP had a basal release, occlusion of the left anterior descending coronary artery only affected SP release, causing an increase from lamina I-VII. Left vagal stimulation increased DYN release, inhibited basal SP release and blunted the CoAO-induced release of SP. DYN release reflected activation of descending pathways in the thoracic spinal cord, since vagal afferent stimulation still increased the release of DYN after bilateral dorsal rhizotomy of T2-T5. These results indicate that electro-stimulatory therapy, using vagal afferent excitation, may induce analgesia in part via inhibition of the release of SP in the spinal cord, possibly through a DYN-mediated neuronal interaction.

Key Words: antibody-coated microprobes, angina, cardiac nervous system, analgesic peptides, nociceptive peptides, substance P, dynorphin
INTRODUCTION

Intractable angina can be one of the more debilitating forms of pain experienced. While myocardial ischemia involves both local changes in myocyte function and reflex alterations in the cardiac nervous system that regulates the myocardium, the perception of angina involves the excessive activation of cardiac sympathetic afferent neurons (16,35). These multi-modal sensory afferent neurons respond to ischemia-induced changes in the chemical/mechanical milieu of the heart (16, 35). The cell bodies of these afferents are found in the dorsal root ganglia of spinal segments C8-T9 (cervical 8-thoracic 9), with the majority associated with spinal segments T2-T6 (29) and project mainly to laminae I, V, VII and X (29). Activated cardiac sympathetic afferent fibers excite cells in the spinal thalamic tract (STT) primarily in T1-T6 spinal segments (5,41) and C1-C2 segments (8), as well as other ascending tracts, including the spinoreticular, spinomesencephalic and spinosolitary tracts (16).

Clinical treatment for ischemic heart disease focuses on relieving the angina through a hierarchy of treatment modalities, starting with life style changes and progressing through pharmacologically-induced coronary vasodilation, angioplasty and finally, coronary bypass procedures. Some individuals, however, are refractory to these procedures and have become candidates for emerging, alternative neuromodulation therapy (32). Neuromodulation therapies include both trans-cutaneous and direct nerve electrical stimulation (32), including low intensity electrical stimulation of left vagal afferent fibers at the cervical-thoracic level (1,42-44,54-55) and spinal cord stimulation (15,16,34). While electro-neuromodulation therapy is not currently widely used clinically in this country for angina, the electrophysiological effects of these approaches have been documented in a number of animal studies (1,7,16,34,42-44). Moreover, clinical experience in Europe indicate that such therapies are effective treatment options for
patients with severe angina who are refractory to conventional anti-anginal therapy and that are not candidates for revascularization therapy (15, 32).

Insights into the basic mechanisms of left vagal stimulation as a treatment modality for angina derive primarily from electrophysiological studies describing changes in the activity of spinothalamic tract (STT) neurons to noxious stimuli (7,44). A number of studies have looked at the changes in electrical activity of cells in the STT that were excited by noxious stimuli and subsequently modulated using stimulation of cervical-thoracic vagal fibers. Ren et al (44) report that the spontaneous activity of 36% of STT neurons was inhibited by all intensities of vagal stimulation. Chandler et al (7) indicate that thoracic vagal stimulation inhibited STT neurons in spinal segments below C3, but excited 46% of the cells in C1-C3. Furthermore, vagal afferent stimulation was found to decrease the cardiac-evoked total motor unit potentials in anesthetized rats (27). Similar electrophysiological changes are reported in response to spinal cord stimulation (16,34).

While much is known about the specific cells and pathways excited by cardiac ischemic-sensitive nociceptive afferent neurons, little information exists regarding specific neuromediator(s) of the nociceptive signal at the spinal level. Recently, we reported that activation of cardiac ischemic afferent neurons by occlusion of the left anterior descending artery induced the release of substance P (SP) from a number of laminae in the thoracic spinal cord (at the T4 level) (23). This led us to propose that heterogeneous activation of cardiac afferents, as occurs during coronary artery occlusion, represents an optimum signal for stimulating neuronal pathways that lead to the perception of angina (23). Further, we proposed that SP serves as a primary nociceptive peptide at the spinal level (23). This suggestion is supported by the following: first, SP co-exists with glutamate in primary afferent c-fibers (9); second, SP is found in high concentrations in the spinal cord and is released during stimulation of visceral and somatic c-fiber afferent nerves (11,13-14,30); third, spinal tachykinin
receptors are activated during nociceptive signaling (10); and fourth, myocardial ischemia activates dorsal root ganglia neurons that are also stimulated by SP (9,24).

Stimulation of left thoracic vagal fibers decreases the activity of many of the cells in the same dorsal horn sites that are excited by activation of cardiac ischemic-sensitive afferent neurons (7,44). Since we showed that SP was released in the thoracic spinal cord in response to cardiac ischemia (23), it was of interest to determine first, whether activation of cardiac ischemic-sensitive afferent neurons also induced the release of an analgesic (i.e., opiate) peptide in the thoracic spinal cord and second, whether electro-stimulatory neuromodulation suppressed the cardiac ischemic-sensitive afferent fiber mediated release of SP in the thoracic spinal cord and third, whether this stimulation induced the release of an analgesic peptide, such as an opiate.

METHODS

Surgical Preparation of Animals. Sprague-Dawley rats (n=27) of either sex (average weight: 328 ± 7 gm) (Harlan, Indianapolis, IN) were anesthetized with urethane (1.5 g/kg body wt, i.p.). Surgical level of anesthesia was maintained via supplemental injections of urethane (30 mg/kg) through the right jugular vein hourly, or more frequently as needed, during the experiment. All procedures and experimental protocols were reviewed and approved by the Institutional Committee on Animal Care and Use and conformed to the Animal Welfare Act according to the Public Health Policy on Humane Care and Use of Laboratory Animals. Body temperature was maintained at 37° C by placing the animal on a heating pad. The left femoral artery was cannulated for arterial pressure measurement and a tracheotomy performed so that the rats could be ventilated with room air using a small animal ventilator (Harvard Instruments) at frequencies of 70-80/min and a tidal volume of 2-3 ml.
The mean arterial pressure (MAP) was calculated from the diastolic pressure plus one-third the pulse pressure. Heart rate (HR) was determined from either the pressure pulse signal and displayed on a Grass chart recorder via a tachograph or calculated from the ECG recording. Data presented in Table 1 are the mean ± SEM.

For hemodynamic data, both the initial response to the experimental intervention as well as the steady-state levels (taken 1 min after initiation of the intervention) are reported in Table 1. Only one experimental intervention (i.e., coronary occlusion or vagal stimulation, etc.) was performed on a given group of rats, with each intervention repeated twice for a total of three interventions for each animal. The group mean reported in Table 1 was derived from the average response of the repeated intervention for each animal. There was no difference in the BP or HR responses for the first compared to the third intervention in any given animal, thus the MBP and HR levels were averaged for each rat and this value was entered into the group mean. Significance was determined using (SigmaStat) the Student’s T-test for paired data within a specific experimental group (for rest versus intervention or intervention versus recovery) and using one-way ANOVA for between group analysis with subsequent follow-up comparisons using Tukey’s test. \( P \leq 0.05 \) is taken as the minimum level of significance.

**Laminectomy.** The spinal cord from T1-T6 was exposed on all the rats used in these experiments by removing the corresponding vertebral processes. This was done to allow placement of the antibody-coated microprobes. An intravenous injection of turbocurarine (67 \( \mu \)g/kg body wt) was given prior to removal of the dura and pia maters from these segments. The exposed spinal cord was kept moist and warm by superfusing sterile, oxygenated, artificial cerebrospinal fluid (in mM: NaCl 125; KCl 3; CaCl\(_2\) 2.5; MgCl\(_2\) 1.2; NaHCO\(_3\) 25; dextrose 3.7; urea 6; BSA 0.1%) over the area, wrapping the area with a piece of plastic film and positioning a heat lamp over the area.
The animal was fixed in a Kopf stereotaxic frame and the spinal cord maintained in a fixed position by the use of spinal clamps secured to the vertical processes of segments both rostral and caudal to the laminectomy. Some of the rats (n=7) also underwent a dorsal rhizotomy. The purpose of the dorsal rhizotomy was to determine whether afferent input from the ischemic myocardium altered the release of an analgesic peptide since we found that thoracic rhizotomy significantly attenuated the release of substance P (see ref 23). In these cases, the lateral processes of T1-T6 were completely removed. The dorsal roots of spinal segments T2-T5 were identified, gently separated and then sectioned bilaterally, close to the lateral most area of each segment. A 60 min rest period was allowed following completion of all surgical procedures before the experimental protocols involving either coronary artery occlusion or left vagal stimulation was initiated (see below). For animals with dorsal rhizotomy, the left vagal stimulation protocol was followed as described below for animals with intact dorsal roots (see Left vagal Stimulation).

**Coronary artery occlusion.** (n=7 rats) A left thoracotomy was performed between the 4th rib following initiation of ventilation. A segment of saline-soaked 5-0 suture was looped around the left anterior descending coronary artery, near its branch point from the left coronary artery. The ends of the suture were passed through a one-inch length of double-barreled polyethylene tubing. The ends of the tubing were rounded so that no rough surface of the tubing would damage the coronary artery. The tubing was gently placed next to the heart and secured by three knots tied on the suture at the external end of the tubing length, with the last knot up against the end of the tubing. The tube and suture assembly was then externalized and the thorax closed. Coronary artery occlusion was accomplished by advancing the last knot up against the external end of the tubing, 2 mm away from the tubing. This permitted reproducible occlusion of the left anterior descending artery without tearing the vessel. Either a DYN- or SP-antibody
coated microprobe was positioned in the T4 spinal level (see below) for 10 min (‘Rest’ probe) followed by a second and then third ‘rest’ probe inserted for subsequent 10 min periods prior to the beginning of the occlusion sequence. Coronary artery occlusion was applied sequentially for 90 sec with a 60 sec rest interval over a 10 min period (i.e., 4 occlusions were applied over the 10 min period). This constituted the coronary occlusion intervention and a single microprobe remained in the spinal cord during this 10 min period. A ‘Recovery’ probe was placed in the spinal cord at the completion of the occlusion intervention for 10 min. After a 30 min rest, the entire coronary occlusion procedure was repeated twice in each animal (with a fresh ‘coronary occlusion’ probe and a fresh ‘Recovery’ probe used for each period).

**Left Vagal Stimulation.** (n=7 rats) Vagal nerves were isolated at the level of the C8-T1 spinal segments and sectioned bilaterally. A platinum bipolar electrode was placed on the central end of the sectioned left vagus and fixed with Kwik-Cast (World Precision Instruments, Inc.). The left vagus was used since it has more afferent fibers than the right (22). Left vagal stimulation was applied by using a Grass constant current stimulator with an isolation unit using 25V, 0.2ms at 10 Hz (1). Stimuli were delivered sequentially for 90 sec with 60 sec rest intervals over a 10 min period (i.e. 4 stimuli trains). The left vagal stimulation procedure was repeated twice in each animal. Separate probes were placed in the thoracic spinal cord for 10 min each during Rest, prior to the electrical stimulation, for 10 min during the stimulation procedure and for the 10 min Recovery period, immediately after the stimulation procedure, as described above for the coronary artery occlusion protocol. For animals with bilateral dorsal rhizotomy (T2-T5), a similar left vagal stimulation protocol was followed.

**Combined Coronary Artery Occlusion and Left Vagal Stimulation.** (n=6 rats) The left anterior descending coronary artery and vagi were prepared as outlined above. Concurrent left anterior descending coronary occlusion and left vagal stimulation were
applied for successive 90 sec intervals with 60 sec rest periods over a 10 min period and repeated twice in each animal. Microprobes were placed in the thoracic spinal cord as described for the other protocols.

*Measurement of immunoreactive SP (irSP) and dynorphin (irDYN) using immobilized antibody microprobe technique.* The release of endogenous irSP and irDYN from sites in the thoracic spinal cord was measured using the antibody-coated microprobe technique, as previously described (12,50). Each probe was inspected to verify that the shafts were straight. Glass microelectrodes (tip diameter ~10 µm; shaft diameter 2mm from the tip, ~20-30 µm) were coated as described before and incubated for 24 hrs at 4°C with protein A (Sigma Chemical) prior to the experiments (50). Probes were then incubated with their respective antibodies (for either SP or DYN A (1-13)) for two 24 hr periods at 4°C in 5µl of a 1:1000 dilution (Phoenix Pharmaceuticals) in PBS-azide solution, pH 7.4, with the solution changed after the first 24 hrs. The SP antibody was determined not to react with neurokinin A, B or K. The DYN antibody had negligible cross-reactivity (≤1.5%) with DYN (1-10) or (1-11). We also tested whether endomorphin-2 was released from the thoracic spinal cord. Antibody to endomorphin-2 (Phoenix Pharmaceuticals, 1:1000) was incubated with microprobes, as described for SP and DYN (cross-reactivity with endomorphin-l, <10%). In all experiments where probes were inserted into the thoracic spinal cord, a set of control probes (designated as *in vitro* probes) was identically and simultaneously prepared as the *in vivo* probes. These *in vitro* probes were used to determine the sensitivity of the binding of radiolabeled ligand (125I-Tyr8 SP or DYN, Phoenix Pharmaceuticals) (see 12 or 50) and to confirm the uniformity of binding of the silane and antibody along the shaft of the probes. Typically, 10^{-7} M SP will displace ~50% of the labeled SP and 5 x 10^{-7} M DYN will displace ~50% of the labeled DYN from approximately a 1 cm length of a probe tip.
Dose response trials indicate that between $10^{-9}$ M and $10^{-10}$ M unlabeled ligand will give statistically different counts than buffer alone when incubated with probes. Each in vivo experiment utilizes probes from different batches so any differences in coating is randomized and the results from no one category or group of probes (e.g., “rest” or “stimulus”, etc.) would be skewed.

Each of the *in vivo* probes was positioned at the midpoint of the T4 spinal segment (in terms of its length) at 0.5mm lateral to the midline and inserted into the cord to a depth of 2 mm below the dorsal surface. Each probe remained *in situ* for 10 min, for the duration of either the rest, the experimental procedure (coronary occlusion and/or left vagal stimulation, as described above) or the recovery periods. New probes were used for each 10 min pre- or post- experimental procedure and each experimental procedure and these were designated as ‘rest’, coronary occlusion (‘CoAO’), left vagal stimulation (‘LVS’), coronary occlusion with left vagal stimulation (‘LVS+ CoAO’) or ‘recovery’. A total of 247 probes were used and analyzed for these experiments. Each probe was calibrated against the midline and surface of the spinal cord and placement of each probe was performed using a stereotaxic surgical microscope to ensure repeatability of their placement. At the completion of the experiment, 9.7-13.4 nl of Pontamine blue dye was deposited through the last probe via an automated positive-pressure injection system (Nanoject II) to verify position of the probe tips. This was done by visualizing the dye in 30 μm cross-sections of the spinal cord at the completion of the experiments, using a stereotaxic atlas (39). The deposit of blue dye was visualized through an Olympus OM-3 microscope and the image of the spinal section captured through the image analysis set-up following distance calibration. The image was then compared to the standardized section in the reference atlas (39).

At the completion of the 10 min *in situ* time, probes were withdrawn from the spinal cord, washed briefly in ice-cold PBS and incubated with radiolabeled SP or DYN
(0.01 µCi/ 5 µl PBS, 7.4) for 24 hr at 4° C. Probes were washed in ice-cold PBS-Tween (0.05% Tween 20, Fisher) for 15 min under vacuum manifold to remove any radioactive solution that may have been on the inside of the probe. A 1 cm length of the probe, starting from its tip was broken off, secured to a piece of heavy paper and counted for radioactive binding. The shaft ends of the tips were secured to a sheet of paper and placed against mono-emulsion x-ray film (Kodak, Biomax MR-1) for 3 days. The set of in vitro probes, incubated with the same amount of radiolabeled ligand, was washed in the Tween-PBS and exposed with the same sheet of x-ray film as the in vivo probes.

Image Analysis of the Microprobes. Autoradiographic images of the microprobes were analyzed for patterns of inhibition of binding of the radiolabeled ligand along the length of the probe. Such inhibition is indicative of where unlabeled (e.g., endogenously released) SP or DYN bound to the antibodies on the probe during the in vivo exposure time. Since the position of the probe-tips was marked by a deposit of blue dye in the spinal cord, sites of release of the peptide could be determined from differences in the optical densities of the probe images on the x-ray film (see Fig 4). The analysis was carried out based on initial methods described by Hendry et al (20) and modified by this laboratory. A computerized image analysis system (MCID, Imaging Research, Canada) was used to linearly integrate the images transversely in 16 µm increments for the total 4 mm length of the probe, starting from the probe tip. Background grayness, due to the exposed x-ray film alone, was subtracted from each pixel of the probe image.

In the diagrams presented, the mean optical density of the probe image is converted to a gray scale in arbitrary units of 0-1026 (with 1026 being the darkest gray level). Each probe image was analyzed for 4 mm: the first 2 mm, starting at the tip, corresponded to the segment of the probe inserted into the spinal cord (designated 2 to 0); while the next 2 mm corresponded to the part of the probe that remained outside the
spinal cord (designated as “0 to -2 mm”). The 2 mm outside the spinal cord served as an internal control area along each probe and for between group controls via comparison to similar segments on the *in vivo* and *in vitro* probes. The data presented in the image analysis figures are given as the mean gray levels ± SEM for each specified group of probes. Differences in the patterns of binding of radiolabeled SP or DYN A (1-13) along the probes during various experimental interventions were determined by Student’s T-test for paired data. The calculated T value, where P=0.05 (the minimum level of significance taken), is plotted along the lower portion of the image analysis graphs (just above the abscissa). The T-value for each pixel along the analyzed image was calculated and plotted in relation to the T-value of P=0.05. Any points along the length of the probes that were different from each other appear above the T-value line and indicate significance. Since the resolution of detecting a difference in the binding of radiolabeled peptide is of the order of 100 µm (12), biological significance was defined only when the difference between two groups (i.e., the T-value) was maintained above the P=0.05 line) for a linear distance of at least 100 µm. Differences which appeared above the t-value line but were <50-100 µm in length were not considered a biological event. This technique determined whether either SP or DYN was released, what specific sites in the spinal cord released these neuropeptides and whether an experimental intervention altered the spatial pattern of release of these peptides within the T4 segment of the spinal cord. This was possible since the tips of the probes were located by identification of the deposited blue dye in the histological sections of the spinal cord using a reference atlas (39). This technique is not used to quantify differences in the amounts of peptides released and this should not be inferred from the data presented (e.g., the T-value above the significance line).
RESULTS

Effects of Coronary Artery Occlusion on DYN Release

Insertion of microprobes into the spinal cord had no effect on blood pressure or heart rate. Applying coronary occlusion caused a modest hypotension and tachycardia, as seen in Table 1. Sham operated animals (for the coronary artery occlusion procedure, n=4) had resting MAP of 122±9 mmHg and HR of 390±21 bpm. While the resting MAP and HR in the coronary artery occlusion group were lower than the sham group, these differences were not significant (MAP, P=0.08, F=3.97; HR, P=0.70, F=0.158, respectively), except for the animals undergoing left vagal stimulation following rhizotomy (see Table 1). Typical autoradiographic images of DYN antibody-coated microprobes are shown in Figure 1. Compared to the \textit{in vitro} control probe (Fig 1F), a probe inserted into the T4 spinal cord during rest periods shows a less dense image from its tip to about 1.5-2.0 mm from its tip (Fig. 1A), indicating inhibition in binding of radiolabeled DYN along this part of the probe. The segment of the probe that remained outside the spinal cord (i.e., the next 2 mm) has a uniform intense image that resembles the appearance of a control \textit{in vitro} probe, as seen in Fig. 1F. Insertion of microprobes during coronary artery occlusion also revealed a less intense binding for the 2mm of the probe that remained \textit{in situ} for the 10 min of intermittent occlusion of the left anterior descending coronary artery (see Fig. 1B) compared to the control \textit{in vitro} probe (Fig 1F).

Summary image analysis graphs showing the average gray levels of rest probes compared to \textit{in vitro} probes are presented in Figure 2A. The gray levels of the 2mm of the rest probes that were in the spinal cord were less intense compared to the gray levels of the same length segment of the control \textit{in vitro} probes (i.e. upward shift), indicating an inhibition in binding of radiolabeled DYN, thus a basal release of endogenous irDYN from laminae I-VII in the dorsal horn. Figure 2B shows the gray levels of the rest probes and the probes in the spinal cord during coronary artery occlusion.
occlusion were not different from each other (i.e., no length of the 2mm of the Rest or coronary artery occlusion probes had T-values greater than P=0.05). This indicates that no additional irDYN was released in response to coronary artery occlusion. Correspondingly, the gray levels of the Rest and Recovery probes were also virtually identical, indicating similar patterns of endogenous DYN release were evident post-coronary occlusion, compared to baseline rest (see Fig. 2C).

Effect of Left Vagal Stimulation on DYN Release

Applying electrical stimulation of the central end of the left vagus at the cervical-thoracic junction in this preparation, with bilateral vagotomy, lowered BP but had little effect on HR (see Table 1). Left vagal stimulation increased the release of irDYN from the thoracic spinal cord above the resting levels. Note the diminished image intensity in Fig. 1C (i.e., the 2mm in situ segment) as compared to a typical rest probe (seen in Fig. 1A). Fig. 3A summarizes the effect of left vagal stimulation and indicates that the vagal stimulation-induced increased release of DYN was evident from 0 mm (i.e., surface of spinal cord) to ~1.2 mm in the spinal cord. This corresponds to laminae I-V and the dorsal aspects of lamina VII as the sites of release of irDYN, as shown in Fig 4. Data from this set of experiments also indicated that the sites of irDYN release corresponded with neuronal soma that stained FOS positive in response to the left vagal stimulation stress (this laboratory, unpublished results). We also tested whether the opiate endomorphin-2 was released from the T4 spinal level in response to left vagal afferent stimulation. While there was a basal release of endomorphin-2 from laminae I-III, V and VII (n=12 Rest probes, n=12 in vitro probes), the LVS did not induce further detectable release of endomorphin-2 (n=12 Rest probes, n=13 LVS probes – graph not shown).
Subsequent experiments evaluated the potential for interaction between left vagal stimulation and coronary artery occlusion for DYN release. In a separate group of rats from those described in Fig. 3A, Fig. 3B shows that the differences in the gray levels between the rest probes compared to the probes during simultaneous coronary artery occlusion and left vagal stimulation occurred along the same segment length (i.e., 0 to ~1.3mm) as the differences observed between rest and left vagal stimulation probes (shown in Fig. 3A). This finding indicates that application of coronary artery occlusion during left vagal stimulation did not alter the sites of release of irDYN from T4 spinal segments. Furthermore, the differences in the gray levels at this same segment length (i.e., 0 to ~1.3mm) are maintained when comparing the binding profiles of the coronary artery occlusion probes to the coronary artery occlusion + left vagal stimulation probes (see Fig. 3C).

In the next group of rats, the left vagal stimulation procedure was performed following bilateral dorsal rhizotomy (dR) of T2-T5 segments. Rhizotomy tended to result in a higher resting BP than the vagal stimulation group but this difference was not significant (P=0.73). While the mean steady-state BP in the vagal stimulation + dR group was higher than the left vagal stimulation alone group, this was not significant (P=0.29) (see Table 1). As seen in Fig.3E, left vagal stimulation still caused less binding of radiolabeled DYN to probes in the thoracic spinal cord. The same differences between the rest probes and the corresponding in vitro probes (graph not shown) occurred for this group of rats as for the control condition shown in Fig. 2A (in rats with intact dorsal roots). These data indicate that dorsal rhizotomy did not affect the basal release of irDYN from the T4 spinal cord. Left vagal stimulation continued to induce DYN release following rhizotomy (see Fig 3D). This finding suggests that the left vagal stimulation is the initiating event causing the release of endogenous irDYN in the thoracic spinal cord. This notion is supported by the data shown in Fig. 3E which
compares the binding patterns of the left vagal stimulation probes during simultaneous coronary artery occlusion and left vagal stimulation. There was no difference between these two groups of probes, again indicting that cardiac ischemia, i.e., coronary artery occlusion, was not the stimulus for increasing irDYN release from the thoracic spinal cord. Furthermore, the binding profiles of probes during left vagal stimulation in rats with dorsal rhizotomy (see Fig. 3F) indicate that left vagal stimulation induces irDYN release, albeit the release was shifted slightly to deeper lamina within the T4 spinal level compared to rats with intact dorsal root afferent input. Together, these data indicate the predominance of descending projections in mediating the spinal cord neuronal release of DYN initiated by stimulating vagal afferent axons during left vagal stimulation.

Effects of Left Vagal Stimulation on SP Release

As observed previously (18), there is a background release of substance P from the thoracic spinal cord (Fig 5A, comparing in vivo rest versus in vitro probes). Left vagal stimulation suppressed this basal release of irSP from the thoracic spinal cord from laminae III-VII (Fig. 5B). Even though coronary artery occlusion increased release of irSP from laminae I-VII (Fig 5 D), applying left vagal stimulation concurrently with coronary occlusion blunted SP release in these regions (Figs 5C, 5D). In addition, the hemodynamic changes (hypotension and tachycardia) accompanying coronary occlusion were mitigated by pre-emptive left vagal stimulation (Table 1).

DISCUSSION

Refractory angina affects a considerable number of individuals. Those who suffer from this condition and have inadequate relief from the pain following classical surgical and/or pharmacological clinical treatment might be candidates for electo-neuromodulation therapy (28,32,33,34). Two such approaches that have been tried are
vagal afferent or spinal cord stimulation (18,28,55-55). Both are reported to reduce intractable angina with no adverse cardiovascular consequences (18,28,32,42,43,54,55). Widespread clinical studies are limited and how such approaches exert their anti-anginal effects is not well defined, but the mechanisms likely involve both cardiac (18,28,54,55) and neuro-modulator (18,28,34,38,55) components. While the ESBY (Electrical Stimulation versus Coronary Bypass) study indicates that neuromodulation therapy is an effective treatment option for patients with chronic refractory angina (15), further and more extensive clinical investigation would be needed to verify the efficacy of these techniques as an anti-anginal therapy. Moreover, additional basic studies are required to determine the underlying targets (central versus peripheral) that are modified by each specific type of neuromodulatory therapy. The systems controlling the cardiovascular responses to activation of cardiac ischemic-sensitive afferent neurons involves both central and peripheral autonomic and intrinsic-cardiac neural adjustments; i.e., there is a hierarchy of synergistic interactions at multi-levels in the central nervous system (2). Electro-modulatory interventions, such as left vagal afferent nerve stimulation most likely impacts the processing of these control mechanisms at the upper cervical spinal cord level (C1-C3) as well as the brainstem (18) and influences the responses to the input signals from the heart at the thoracic spinal (T2-T6) level (18).

There are a number of electrophysiological studies that show cervical-thoracic vagal afferent stimulation inhibits spinothalamic tract neurons (STT) not only in the thoracic spinal cord in response to cardiac ischemia (1,8,40), but also in the lumbar and sacral spinal levels in response to viscerosomatic noxious stimuli (1,8,16,17,18,19,40). There is, however, virtually no information about the neurochemical mediation of visceral afferent nociceptive signaling or its modulation by electro-stimulatory techniques. This study offers the following major findings: first, that activation of cardiac ischemic-
sensitive afferent neurons by coronary artery occlusion increases release of irSP from the thoracic spinal cord (at T4); second, that coronary artery occlusion in and of itself, does not increase detectable changes in the release of DYN from T4; third, that left vagal afferent axonal stimulation inhibits the release of SP from the thoracic spinal cord; fourth, left vagal afferent stimulation applied during coronary artery occlusion blunts cardiac afferent-mediated release of SP from the thoracic spinal cord; and fifth, left vagal stimulation increases the release of DYN from the thoracic spinal cord and this release is likely mediated via descending pathways into the thoracic spinal cord. The origin of this descending projection, whether from higher cervical spinals segments or supraspinal sites, remains to be determined. Taken together, these data indicate that the anti-anginal effect of electro-modulation with vagal afferent stimulation is mediated in part within the spinal cord by a neuronal mechanisms involving the suppression of SP release by the endogenous opiate, dynorphin A.

Differential activation of left ventricular nociceptive afferent neurons by transient focal ischemia increases the release of the putative neurotransmitter SP which, in turn, may activate STT cells in the thoracic spinal cord (23). STT neurons project to upper cervical spinal segments as well as higher centers in the brain to process the pain associated with myocardial ischemia (16,35). Cardiac ischemic-sensitive afferent neurons are primarily a subset of sympathetic afferent nerves that enter the spinal cord at the thoracic level, with a concentration at the T1-T4 spinal segments (16,18). However, other cardiac ischemic-sensitive afferent neurons are vagal afferents that project to NTS neurons that, in turn, influence neurons in the C1-C3 spinal segments (16). There are a number of descending inhibitory pathways that regulate the activity of neurons in the thoracic spinal cord (16,35) including one that originates from the NTS (16,35). One important pathway that may be relevant to the study conducted here
involves excitation of upper cervical cells, via propriospinal circuits, that stimulates interneurons, which inhibit neurons in the thoracic (and lower) spinal segments (16,18).

Previous results from this laboratory showed an extensive number of Fos-positive cells in the cervical as well as thoracic spinal cord in response to coronary artery occlusion (unpublished results). The majority of the cells activated were found in laminae I-VII and X and more cells were activated in the medial portions of the dorsal horns than the lateral portions. In the current study, we also observed a basal, resting release of irSP from the dorsal horn sites in the thoracic spinal cord and as we reported previously, coronary artery occlusion increased such release (23). Dorsal rhizotomy of spinal segments T2-T5 eliminated the release of SP during subsequent coronary artery occlusion (23). This led us to propose that focal ischemia activated cardiac ischemic-sensitive afferent neurons with subsequent neuronal release of SP into the thoracic cord and such release was a fundamental first step in information signal processing of the ischemic event and ultimately in the perception of angina (23).

This current study indicates there is a basal release of irDYN A (1-13) from a broad area of the thoracic spinal cord at T4. Other studies also report a basal release of DYN peptides from spinal cord sites (25, 45). Our data also show that while coronary artery occlusion produces no change in DYN release from the T4 spinal level by itself, activation of left vagal afferents increases its release, primarily via descending projections. Interestingly, dorsal rhizotomy exacerbates left vagal stimulation-induced DYN release at T4 and differentially shifts that release to deeper lamina of the dorsal horn. These data indicate the dynamic interactions between different levels of the neuroaxis in processing of thoracic afferent inputs. In the context of the well-recognized role of opiates in modulation of pain perception (45,47,53) these data suggest that regulation of neural release of dynorphin within the spinal cord is multifaceted and as
demonstrated in the current study, such release may have profound effects on information transfer of primary visceral nociceptive sensory inputs.

Stimulation of the central end of the left vagus inhibited the basal release of SP from sites in the T4 spinal cord in laminae III-VII and blunted the release of SP when coronary artery occlusion was applied during this stimulation. Electrophysiological studies have shown that chemical or electrical stimulation of vagal afferent fibers increases activity in a subset of neurons in the C1-C2 spinal segments (35,40). It is also known that vagal afferent neuron stimulation decreases the electrical activity of upper thoracic spinal neurons (1). Foreman has proposed that the integration that occurs within this C1-C2 spinal relay accounts in part for the vagal inhibition of the thoracic neurons (1,18). For example, disruption of the circuitry in the C1-C2 relay by the excitotoxin, ibotenic acid, eliminated the inhibition of vagal stimulation on thoracic spinal neurons (56). The results from these experiments (56) and others from Foreman’s laboratory group indicate that the inhibitory effects on thoracic neurons by vagal stimulation does not require direct descending pathways from supraspinal nuclei but rather that propriospinal nuclei in the upper cervical spinal cord are excited by vagal stimulation and the excitation of this pathway accounts for a large part of the suppression of thoracic nuclei activity (6,7,56). Given these findings from Foreman’s group and our laboratory, suppression of the irSP release both at rest and during coronary artery occlusion while left vagal stimulation was applied may also involve propriospinal pathways activated in the cervical segments.

It is well documented that a major source, although not the exclusive source, of SP in the superficial laminae of the spinal cord comes from primary afferent endings (3,31,46) and previous studies involving activation of cardiac ischemic-sensitive afferent neurons and the effects of thoracic dorsal rhizotomy support this concept (23). SP containing intrinsic interneurons (segmental and intersegmental) can also serve as the
source of the SP measured with the antibody microprobes. Since left vagal stimulation alone inhibited the basal resting release of irSP from laminae III-VII, the source of the resting SP could have come from intrinsic interneurons.

Left vagal afferent stimulation may not only excite cells directly in the upper cervical spinal cord, but may also activate supraspinal pathways that contribute to the inhibition of the thoracic dorsal horn sites involved with the mediation of the cardiac ischemic-sensitive signal. Ren et al (43) suggest that propriospinal neurons in the upper cervical spinal segments are excited from a subcoeruleus/parabrachial pathway. Neurons in this pathway, in turn, are activated by vagal stimulation. Alternately, since it is well documented that vagal afferent information synapses directly into the NTS, some of the inhibitory effects of vagal stimulation may be due to activation of descending pathways from the NTS to the cervical spinal cord (16,38). The NTS has a high concentration of SP immunoreactivity (48) and projections from NTS sites via relays either through the pons or rostroventrolateral medulla (42, 43) may transmit an inhibitory signal to the spinal cord.

Our data here show there is a basal release of irDYN A (1-13), as has been reported previously (25,45). We report herein that irDYN A release is increased by vagal stimulation from laminae I-II and from laminae IV-VI. While coronary occlusion itself did not elicit the release of irDYN, coronary occlusion together with vagal stimulation caused an increase in the release of DYN from these same laminae, an effect that was clearly the result of the left vagal stimulation. Dorsal rhizotomy of the afferents entering T2-T5 had no effect on the sites of basal release of this irDYN, yet it did differentially augment irDYN release towards deeper lamina in the T4 segment. Taken together, these data suggest that the source of irDYN released in response to vagal stimulation is from either higher spinal segments (possibly C1-C3 via propriospinal descending pathways) or from supraspinal sites. This finding supports previous studies
that showed that most of DYN immunoreactivity occurred in spinal neurons with a portion derived from primary afferent fibers (36, 49), and that dorsal rhizotomy did not alter spinal cord irDYN levels in rats (4). When comparing the binding profiles of the SP-antibody microprobes during coronary artery occlusion and coronary artery occlusion together with vagal stimulation, these same areas (lamina I-II and IV-VI) appear to be the site of difference between the two conditions.

Our findings demonstrated a functional correlation between DYN release and inhibition of SP release. While we cannot at this point, exclude the possibility that other opiates may be involved with the attenuation of SP release, we do know that endomorphin-2 was not released in response to either coronary occlusion or vagal afferent stimulation. Thus, we now suggest that the increase in release of DYN A (1-13) at the thoracic level by vagal stimulation may be in part or wholly responsible for the inhibition of SP release. While the concept that opioids reduce transmitter release from nociceptive primary afferents is not new, especially with regard to SP (20, 26, 37, 51-53), the data we present here are the first evidence that the interaction between the two peptides may account for the attenuation of the noxious signal activated by coronary occlusion during vagal stimulation.

In summary, these data suggest that left vagal afferent-mediated electro-stimulatory therapy induces its analgesic effect in part via inhibition of SP release within the thoracic cord and that such inhibition involves a DYN-mediated effect on the spinal cord circuits processing primary cardiac afferent neuronal inputs. Correspondingly, left vagal afferent stimulation blunted the autonomic reflexes accompanying transient coronary artery occlusion. These data indicate the interdependent interactions of peripheral and central (spinal and supraspinal) neural mechanisms in the integrated response to myocardial ischemia and its pathological consequences.
ACKNOWLEDGEMENT

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REFERENCES


### Table 1

**Cardiovascular Responses to Vagal Afferent Fiber Stimulation**

<table>
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<tr>
<th></th>
<th>MAP (mmHg)</th>
<th>HR (bpm)</th>
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<tbody>
<tr>
<td></td>
<td>Rest</td>
<td>Initial</td>
</tr>
<tr>
<td>CoAO (n=7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>99±7</td>
<td>95±7</td>
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<tr>
<td>LVS (n=7)</td>
<td></td>
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<tr>
<td></td>
<td>124±5</td>
<td>98±7*</td>
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<tr>
<td>CoAO+LVS (n=6)</td>
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<tr>
<td></td>
<td>122±6</td>
<td>129±12</td>
</tr>
<tr>
<td>LVS+dR (n=7)</td>
<td></td>
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<tr>
<td></td>
<td>136±11*</td>
<td>122±12*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM

n = number of rats in group

*P ≤ 0.05 from corresponding rest

*P ≤ 0.05 from CoAO

CoAO, coronary artery occlusion of the left anterior descending artery

LVS, left vagal afferent stimulation

CoAO+LVS, simultaneous coronary artery occlusion with left vagal afferent stimulation

LVS+dR, left vagal afferent stimulation following dorsal rhizotomy of spinal segments T2-T5
FIGURE LEGENDS

**Figure 1.** Pseudocolor microprobe images. Images of DYN-antibody coated microprobes as they appeared from exposed x-ray film. Pseudocolor automatically applied to the gray levels of autoradiographic probe images converted from optical densities. All probes viewed with tip ends facing leftward. All in vivo probes were inserted into T4 spinal segment 0.5 mm lateral to the mid-line and to a depth of 2 mm. A total length of 4 mm was analyzed (full 4 mm image presented in each panel): 2 mm from the tip inside the spinal cord and the immediate 2 mm that remained outside the spinal cord. Corresponding regions were analyzed on matched in vitro probes. A. Probe inserted into T4 at rest, prior to coronary occlusion. Note the difference in intensity of the image in the first 2 mm of the probe compared to the in vitro control probes (panel F). B. Probe inserted during coronary artery occlusion, CoAO. C. Probe inserted in T4 during left vagal stimulation, LVS. D. Probe inserted during CoAO plus LVS. E. Probe inserted during LVS following dorsal rhizotomy (dR) of spinal segments T2-T5. F. Example of control in vitro probe not inserted into the spinal cord. Scale bar in F: 1 mm (applies to all panels). Arrowheads in A-E: to the left indicates part of the probe that was in the T4 spinal cord; to the right, the part of the probe that remained outside the spinal cord. Note similarities of the outside segments (right-side 2 mm) of the in vivo and in vitro probes. Probe images in A, B and F from same experiment. Probe images in C, D and E from different rats and different experiments, as indicated in the panels.

**Figure 2.** Comparison of the binding patterns of radiolabeled DYN to microprobes and the resulting differences in optical density (converted to gray level in arbitrary units, a.u., with 1026 being the darkest) of autoradiographic probe images. Gray
levels are given as the mean ± S.E.M. (dotted traces above and below the main traces) and are plotted against the length of probe inserted into the spinal cord (2 to 0 mm) together with the 2 mm length that remained outside the spinal cord (0 to –2 mm). Plots of the T-value (solid blue line, P=0.05 minimum level of significance) are superimposed just above the abscissa in each panel. Areas along the length of the probes where the difference in the mean gray levels were equal to or greater than P=0.05 indicate significant differences between the corresponding two sets of probes. A. Comparison of the rest probes, prior to coronary artery occlusion, CoAO to the in vitro probes; B. Comparison of rest probes and probes inserted into the spinal cord during CoAO and C., rest probes compared to recovery probes post-CoAO.

Figure 3. Image analysis graphs of DYN microprobes during LVS ± CoAO procedures. A., Comparison of the binding patterns of rest probes to probes inserted into T4 during left vagal stimulation, LVS; B., rest probes to probes in the spinal cord during simultaneous left vagal stimulation and coronary artery occlusion, CoAO + LVS; C., probes during CoAO to probes during CoAO + LVS; D., rest probes following dorsal rhizotomy, dR compared to probes during LVS after dR (LVS+dR); E, probes during LVS compared to probes during CoAO applied during LVS (CoAO+LVS); and F., probes during LVS compared to probes during LVS following dR (LVS+dR). Details of graphs described in Figure 2.

Figure 4. Sites of release of DYN Projection drawing of T4 spinal segment showing boundaries of dorsal horn laminae superimposed through projection drawings using stereotaxic atlas (37) as reference. The Student’s T-test from Fig.
3A, showing the difference in image analysis graphs between the rest and vagal stimulation (LVS) probes, are superimposed on the section. The t-test is displaced to the right of the midline for illustrative purposes so that sites within the dorsal horn where the differences between these two sets of probes occurred could be identified. Areas showing release include the superficial laminae I-III, V and VII. Pseudocolor image of DYN-antibody coated microprobe during LVS is superimposed to the right of the section for illustrative purposes as well, and its location in the diagram is not representative of location of probes inserted into the spinal cord to capture peptide release. Note the lower intensity binding of radiolabeled DYN, corresponding to the area along the length of the probe where there were significant differences in the grey levels between the Rest and LVS probes. Average location of probe tips within the T4 segment represented by the • symbol (vertical and horizontal bars = SEM, n=27); dashed line indicates position of probes within the spinal segment. Scale bar = 0.5mm (applies to spinal segment, probe image and t-test).

**Figure 5.** Image analysis graphs of SP microprobes. A. Comparison of the rest probes inserted into T4 to in vitro probes; B., rest probes to probes inserted into the spinal cord during left vagal stimulation, LVS; C., rest probes to probes inserted into T4 during simultaneous coronary artery occlusion and vagal stimulation, CoAO+ LVS and D., probes during CoAO to probes during CoAO+ LVS.