Myocardial ischemia induces the release of substance P from cardiac afferent neurons in rat thoracic spinal cord

Fang Hua, Brian A. Ricketts, Angela Reifsteck, Jeffrey L. Ardell, and Carole A. Williams. Myocardial ischemia induces the release of substance P from cardiac afferent neurons in rat thoracic spinal cord. Am J Physiol Heart Circ Physiol 286: H1654–H1664, 2004. First published December 18, 2003; 10.1152/ajpheart.00906.2003.—Antibody-coated microprobes were inserted into the thoracic (T3–4) spinal cord in urethane-anesthetized Sprague-Dawley rats to detect the differences in the release of immunoreactive substance P-like (irSP) substances in response to differential activation of cardiac nociceptive sensory neurons (CNAN). CNAN were stimulated either by intrapericardial infusion of an inflammatory ischemic exudate solution (IES) containing algogenic substances (i.e., 10 mM each of adenosine, bradykinin, prostaglandin E<sub>2</sub>, and 5-hydroxytryptamine), or by transient occlusion of the left anterior descending coronary artery (CoAO). There was widespread basal release of irSP from the thoracic spinal cord. Stimulation of the CNAN by IES did not alter the pattern of release of irSP. Conversely, CoAO augmented the release of irSP from T3–4 spinal segments from laminae I–VII. This CoAO-induced irSP release was eliminated after thoracic dorsal rhizotomy. These results indicate that heterogeneous activation of cardiac afferents, with foci cardiac artery occlusion, represents an optimum input for activation of the cardiac neuronal hierarchy and for the resultant perception of angina. Excessive stimulation of cardiac nociceptive afferent neurons elicited during regional coronary artery occlusion involves the release of SP in the thoracic spinal cord and suggests that local spinal cord release of SP may be involved in the neural signaling of angina.

antibody-coated microprobes; coronary artery occlusion; algogenic substances; angina; cardiac nervous system

MYOCARDIAL ISCHEMIA elicits a complex sequence of events involving local changes in myocyte function and reflex alterations in the cardiac nervous system that controls them. In this regard, myocardial ischemia activates cardiac sensory neurites associated with afferent axons coursing centrally in both sympathetic and parasympathetic nerves (14, 25). Activation of both cardiac afferent populations contributes to the integrated cardiovascular reflex response to cardiac ischemia (14, 25). Conversely, the perception of angina is primarily mediated as the result of excessive activation of the cardiac afferent neurons with somata in dorsal root ganglia that transduce local changes in the cardiac mechanical and chemical milieu (14).

Myocardial ischemia alters the mechanical and chemical milieu of the heart, thereby altering activity as transduced by the associated cardiac sensory neurons. The extracellular concentration of several substances increases, including bradykinin, substance P (SP), prostaglandins, leukotrienes, lactate, potassium, and adenosine. These substances alter the activity generated by a subpopulation of cardiac sensory neurons (2, 14, 21, 26). Intrapericardial infusion of a solution containing one or more of these algogenic substances can change the electrical activity of superficial and deep cells in spinal segments C1–2 and T1–6, the segments where sympathetic cardiac afferent fibers enter (4, 7, 14, 25, 28). Transient myocardial ischemia similarly alters the activity generated by neurons in these same spinal segments (14, 25).

Sympathetic afferent fibers are associated with the myocardium itself and many of their endings project close to the epicardial surface, including the left ventricular wall (25). These are distinct from the afferent neurons that innervate the pericardial sac whose afferent axons course in the phrenic nerve (22). The latter are not involved with the nociceptive signal that accompanies angina (25). Transient occlusion of the left anterior descending coronary artery, which supplies the septum and anterior aspect of both ventricles, activates cardiac sympathetic afferent neurons (2, 6, 18). The cell bodies of these afferents are found in the dorsal root ganglia of C8–T9, with the majority associated with spinal segments T2–T6 (23). Their central projections terminate mainly in laminae I, V, VII, and X (23). Electrical and chemical stimulation of the cardiac sympathetic afferent neurons excites cells in the spinohalamic tract as well as other pathways of the T1–6 spinal segments (4). In contrast, stimulation of cardiac vagal afferent fibers excites neurons primarily in the spinohalamic tract in C1–C3 spinal segments (6) as well as projecting centrally to the nucleus tractus solitarius (25).

Although specific cells and pathways excited by these cardiac nociceptive afferent neurons (CNAN) are well documented, there is minimal information identifying the specific neuromediator(s) of the nociceptive signal at the spinal level. There is supportive evidence for the role of SP as a modulator of angina. Specifically, SP, along with glutamate, coexists in primary afferent c-fibers (8). SP is found in high concentrations in the spinal cord, and it is released at the spinal level during stimulation of visceral and somatic c-fiber afferent nerves (9, 12, 24, 30, 37, 38). Moreover, spinal tachykinin receptors are activated during induction of somatic nociceptive reflexes (9). Finally, myocardial ischemia activates subpopulations of dorsal root ganglia neurons that are also stimulated by SP (8, 19). To determine the potential role of SP as a mediator of cardiac nociception, the objective of this study was to evaluate the pattern of its release at the thoracic spinal level in response to activation of CNAN. Specifically, with the use of the antibody
microprobe technique, we determined that SP release within the spinal cord is a function of the pattern of cardiac afferent neuronal stimulation. We hypothesize that heterogeneous activation of cardiac afferent neurons, as it occurs with focal coronary artery occlusion, represents an optimum input for activation of the cardiac neuronal hierarchy and perception of angina.

MATERIALS AND METHODS

Surgical preparation of animals. Sprague-Dawley rats of either sex (average wt: 264 ± 9 g) (Harlan; Indianapolis, IN) were used in these experiments. All procedures and experimental protocols were reviewed and approved by the East Tennessee State University Institutional Committee on Animal Care and conformed to the Animal Welfare Act according to the Public Health Policy on Humane Care and Use of Laboratory Animals. Rats were anesthetized with urethane (1.5 g/kg body wt) that was injected intraperitoneally; the right jugular vein was cannulated and surgical level anesthesia was maintained via supplemental injections (30 mg/kg) through that vein hourly or more frequently as needed during the experiment. The left femoral artery was then cannulated for arterial pressure measurement, and a tracheotomy was performed. All rats were ventilated using a small animal ventilator (Harvard Instruments) at frequencies of 70–80/min and tidal volume of 2 to 3 ml. 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. Body temperature was maintained at 37°C by placing the animal on a heating pad.

HR and blood pressure (BP) data are presented as means ± SE. Data are reported for baseline at the steady-state level during each experimental intervention and during the recovery phase. Only one experimental intervention was performed on a group of animals (intrapericardial infusion, coronary artery occlusion, or dorsal rhizotomy plus coronary artery occlusion), with each intervention being repeated twice and the group average derived from the average for each animal. Significance was determined from Student’s t-test for paired data within a specific experimental group and using one-way ANOVA between groups. P ≤ 0.05 is taken as the minimum level of significance.

Intrapericardial infusion of ischemic exudate solution. In group 1, a midsternal incision was made and the thymus was separated at the midline for a short length. This made a small opening into the pericardial sac into which a short length of silicone tubing (0.25 in. ID, 0.47 in. OD) was inserted and advanced along the surface of the heart toward the apex. The catheter was filled with 0.2–0.5 ml of warmed normal saline and flushed regularly throughout the rest period. The experimental intervention consisted of intrapericardial infusion of the ischemic exudate solution (IES) consisting of 10 mM each of adenosine, bradykinin, prostaglandin E2, and 5-hydroxytryptamine. For these infusions, a 0.2-ml volume of IES was infused through the catheter for 2 min, followed by a washout with normal saline every 2 min, for a total of 3 infusions every 10 min. This was followed by a 10-min recovery period when fresh warmed saline was infused through the catheter. After a 30-min rest period, this entire procedure was repeated in every rat. Controls for the IES utilized repeated time-matched normal saline infusions into the intrapericardial sac. Cardiovascular data were averaged for each animal before being included with the group data from other animals. At the completion of the experiments, 0.2 ml of Pontamine blue dye was infused through the catheter to verify the solution remained within the pericardial sac. SP antibody microprobes were inserted into T4 spinal segments at either 0.4–0.5 mm lateral to the midline (n = 13) or 0.7–0.8 mm lateral to midline (n = 6).

Coronary artery occlusion. In group 2, a left thoracotomy was performed at the fourth rib. A segment of saline-soaked 5-0 suture was looped around the left anterior descending coronary artery, near its origin from the left coronary artery. The ends of the suture were passed through a 1-in. segment of double-barreled polyethylene tubing. The ends of the tubing were rounded so that a smooth surface abutted 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 tubing and suture assembly was then externalized and the thorax closed. Occlusion of the left anterior descending coronary artery (CoAO) was accomplished by withdrawal of the distal knot 2 mm away from the tubing end. This permitted reproducible occlusion of the left anterior descending coronary artery without tearing the artery. The CoAO was applied for 60 s with a 60-s rest interval over a 10-min period (thus 4 occlusions were applied over a 10-min period). After a 30-min rest period, the CoAO procedure was repeated in each animal. The data were handled the same as described for the IES infusions.

For group 3, animals underwent a dorsal rhizotomy (DR) before excitation of CNAN. During this procedure, thoracic spinal segments T1–6 were exposed and their lateral processes removed. An intravenous injection of turbocurarine (67 μg/kg body wt) was given before removal of the dura and pia mater(s) from these segments. 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 to occur after completion of all surgical procedures before the experimental protocol involving CoAO was initiated. The CoAO protocol was the same as described for the group 2 control animals, with two successive occlusions.

Measurement of immunoreactive SP-like substances using immobilized antibody microprobe technique. The release of endogenous immunoreactive (ir)SP-like substances from sites in the thoracic spinal cord was measured using the antibody-coated microprobe technique (11), as previously described by this laboratory (34, 35). Antibodies to SP (Phoenix Pharmaceuticals) were immobilized to the outer surface of glass microelectrodes by incubating the probes for two 24-h periods at 4°C before the experiments in a 1:1,000 dilution of the antibody in a PBS-azide buffer, pH 7.4. This antibody did not cross react with neurokinins A, B, or C. A set of control probes (designated as in vitro probes) were identically and simultaneously prepared as the in vivo probes, and these were used to not only to determine the sensitivity of the binding of radiolabel SP to each batch of probes but also to confirm uniformity of binding of the silane and antibody along the shaft of the probes. These in vitro probes were incubated in a time-matched exposure with buffer alone (PBS-azide, pH 7.4) and then incubated with the radiolabeled SP, as described below for the in vivo probes.

At the T4 spinal segment, each in vivo probe was positioned 0.5 mm lateral to the midline to a depth of 2 mm below the dorsal surface. Placement of each probe was performed using a stereotaxic surgical microscope to ensure accuracy and repeatability. Each probe remained in situ for 10 min, and separate probes were used for each sequential rest, intervention (IES or CoAO), and recovery period. 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) to verify position of the probe tips. Verification was carried out by visualizing the location of the dye in 30 μm cross-sections of the spinal cord at the completion of the experiment. The site where the dye was found was identified with the use of a stereotaxic atlas (27) as a reference.

At the completion of each sequential 10 min in situ time, probes were withdrawn from the spinal cord, and each was incubated with 125I-labeled SP (0.01 μCi/5 μl) for 24 h at 4°C, then processed as described before (34, 35). Differences in SP binding were determined by image analysis of the resultant X-ray autoradiographs.

Image analysis of microprobes. The images of the microprobes on the X-ray film were analyzed for patterns of inhibition of binding of radiolabeled SP. Such inhibition indicates the points along the probe where endogenously (unlabeled) SP bound to the antibodies along the probe. The analysis was carried out based on methods described by Hendry et al. (16) as modified by this laboratory (34, 35). A comput-
erized image analysis system (MCID, Imaging Research) was used to integrate the images for a total length of 4 mm: the first 2 mm, starting at the tip, corresponded to the segment of the probe inserted into the spinal cord (designated 0 → 2 mm), whereas the next 2 mm corresponded to the part of the probe that remained outside the spinal cord (designated 0 → −2 mm). The 2 mm outside the spinal cord served as an internal control area along each probe and also functioned as a control for potential interprobe variations. In vivo and in vitro probes were analyzed and compared along the entire 4 mm probe exposure. 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 was converted to a gray scale in arbitrary units of 0–1.026 (with 1.026 being the darkest gray level). The data are given as the mean gray levels ± SE of specified groups of probes. Differences in the patterns of binding of radiolabeled SP along the probes during various experimental conditions were determined by Student’s t-test for paired data. The calculated T value, where \( P = 0.05 \) (the minimum level of significance), is plotted along the lower portion of the image analysis graphs (just above the x-axis). The T value for each pixel along the analyzed image was calculated and plotted in relation to the T value of \( P = 0.05 \). For between-group comparisons, any points along the length of the probes that were different from each other applied the T-value line (i.e., the positive and negative T-value line; see Figs. 3, 4, and 7) and indicate significance. Because the resolution of detecting a difference in the binding of radiolabeled peptide is \( \sim 100 \mu m \) (11), we defined biological significance only when the difference between matched probe groups exceeded \( P = 0.05 \) for at least 100 \( \mu m \) of probe length. Because the probe location was verified by the deposited dye in the histological sections of the spinal cord using a reference atlas of Paxinos and Watson (27), this technique determined what spinal cord sites released SP and whether an experimental intervention altered the pattern of that release.

**Fos and SP immunohistochemistry.** After completion of the experimental period, some animals were given a large dose of urethane (0.5 ml) and then perfused transcardially with 500 ml of normal saline using a peristaltic pump (Preston Manostat), followed by 500 ml of 4% paraformaldehyde in PBS (pH 7.4) for determination of either c-Fos or SP immunostaining. A block of thoracic spinal cord was transferred once in PBS with 0.4% Triton X-100 and then aluminum foil to eliminate exposure to light. Sections were rinsed again twice in PBS and then finally once in PBS with 0.4% Triton X-100 and 0.25% BSA. Sections were mounted on slides and coverslips applied with the use of Vectashield (Vector Labs; Burlingame, CA) and then placed in a light tight box until viewed. Sections stained for Fos were viewed with the use of an Olympus OM-2 microscope with bright field. SP-FITC-stained sections were viewed with a Leica inverted confocal microscope (DMI2B) at a wavelength of 488 nm and documented using Leica confocal software (version 2.5-0871).

**RESULTS**

**Intrapericardial infusion of IES.** Resting blood pressures remained stable over an entire experimental period, as shown in Fig. 1. A and B. Insertion of SP-antibody-coated microprobes into the thoracic spinal cord had no effect on resting arterial pressure or heart rate, as seen in Fig. 1. Infusion of normal saline intrapericardially did not alter either the mean BP (MBP) or HR from resting levels, as shown in Fig. 1 and Table 1. Initial infusion of IES tended to produce a transient depressor and bradycardic response (see Fig. 1D), and, overall, over the 10 min of coronary occlusion stress, these changes were not significantly different from resting levels (see Table 1).

Representative images of SP-antibody-coated microprobes under various experimental conditions are shown in pseudocolor in Fig. 2. The uniformity of the color density reflects the uniformity of binding of the radiolabeled SP to the probe. The in vitro probe (Fig. 2F), which was never inserted into the spinal cord, demonstrates a uniform binding along the shaft of the probe compared with the rest probe (Fig. 2A) that was inserted into the spinal cord prior to infusion of IES. Note that the 2 mm of the probe (from its tip) that was inserted into the spinal cord exhibited a reduced binding intensity. Conversely, the 2 mm of the rest in vivo probe that remained outside the spinal cord was similar to the in vitro probe. A similar pattern (i.e., reduced radiolabeled SP binding from the 2 mm of microprobe inserted into the spinal cord) was also evident for the probe inserted during IES infusion (Fig. 2B).

The summary binding patterns of the probes inserted into the thoracic spinal cord at T4 at 0.4–0.5 mm lateral to midline, during the IES stressor is shown in Fig. 3. At baseline, note there is a significant difference in the average gray levels between the 2 mm length of the in vivo “rest” microprobes that were in the spinal cord and the in vitro probes, with the rest probes having lower average gray levels (along the 2 mm that was in the spinal cord) than the in vitro probes. These data indicate that there was a basal release of irSP during rest. Endogenous SP release was widespread and occurred from...
superficial lamina I to laminae VI–VII. Intrapericardial infusion of normal saline did not alter the binding of radiolabeled SP to microprobes (see Fig. 3B) compared with the rest probes. The binding patterns of the “rest” probes to the probes inserted into the spinal cord during the IES infusion likewise showed virtually identical gray levels (Fig. 3C) along their entire length, for both the 2 mm placed in the spinal cord and the 2 mm that remained external to the cord. This indicated that there was no difference in the pattern of SP release during IES infusion compared with baseline. Finally, the binding patterns of the postinfusion, “recovery,” probes were also similar to rest (Fig. 3D), suggesting that the levels of irSP remained stable throughout the experimental period. The same results were obtained when microprobes were inserted 0.7–0.8 mm lateral to the midline (see Fig. 4). This was done because studies with Fos immunostaining showed that CNAN stimulated by the IES procedure excited more neurons in the lateral portion of the dorsal horn in the thoracic segments (F. Huas and C.A. Williams, unpublished results). IES infusion did not elicit any further release of SP from these more lateral sites either, as seen in Fig. 4B.

**Activation of CNAN by CoAO.** CoAO resulted in an immediate decrease in BP that remained depressed as long as the occlusion was applied. The decreased MBP was significantly lower than rest levels and recovered on release of the occlusion.

![Fig. 1. Chart recordings showing changes in blood pressure (in mmHg) and heart rate [beats/min (bpm)] during various procedures. A: placement of substance P (SP)-antibody coated microprobe (designated by ● in A–E) in spinal segment T4 during initial rest period of the control experiment. B: placement of microprobe in same animal 3 h after initial probe placement. C: effect of intrapericardial infusion of normal saline (initiated at ■ “in”) and withdrawal of saline (■ “out”). D: effect of initial intrapericardial infusion of inflammatory ischemic exudate solution (IES) (■), subsequent infusion of saline and repeated IES (placement of microprobe designated by ●). E: effects of applying and removing the transient occlusion of the left anterior descending coronary artery (CoAO) (■) in an animal with intact dorsal roots and F: applying and removing CoAO (■) in a different rat after dorsal rhizotomy of T2–T5 roots. Overall, blood pressure (BP) was lower in animals undergoing dorsal rhizotomy (dR).](http://ajpheart.physiology.org/)

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<th>Table 1. Cardiovascular changes during activation of CNAN</th>
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Values are means ± SE; n, no. of rats per group. CNAN, cardiac nociceptive sensory neurons; IES, inflammatory exudate solution; CoAO, transient occlusion of left anterior descending coronary artery; dR, dorsal rhizotomy. Saline was injected intrapericardially. *P ≤ 0.01 from corresponding rest in same group; †P ≤ 0.01 from CoAO (intact dorsal afferents) group.
This change in MBP was associated with a slightly higher but not significant change in HR (see Table 1) during occlusions.

The pseudocolor images of the probes inserted during rest before coronary occlusion and during CoAO are shown in Fig. 2. There is no difference between the 2 mm of the rest (Fig. 2C) or CoAO (Fig. 2D) probes that remained outside the spinal cord to each other or compared with the in vitro probe, indicating a similarity in binding of the radiolabeled SP to this segment of the probes. However, the binding along the in situ segment of the CoAO probe (Fig. 2D) is reduced from the corresponding rest probe (Fig. 2C). These data indicate an induced increase in release of endogenous SP during the coronary artery occlusion.

The summary binding patterns of the probes, at rest, during CoAO and recovery are presented in Fig. 5. The average gray level of the rest probes, from their tip along the 2 mm of these probes that was in the spinal cord, was reduced from that of the in vitro probes. As with the IES group, these animals demonstrated a basal SP release from superficial lamina I through lamina VI–VII. Moreover, the average gray levels of these resting probes were not different from the average gray levels of the resting probes inserted into the spinal cord before IES infusion, indicating the overall stability and consistency between preparations. However, in marked contrast to the IES challenge, CoAO induced a significant change in endogenous SP release above basal levels compared with the similar segment of the rest probes, as shown in Fig. 5B (i.e., tip at 2 to 0 mm on probe). The induced change in SP release rapidly reversed itself as indicated by the similarities between rest and recovery release profiles (Fig. 5C). This finding is supported by a comparison of the mean gray levels between the CoAO probes and the recovery probes, which showed a significant difference between these levels for the 2-mm segment of probes inserted into the spinal cord (Fig. 5D).

To demonstrate that sites of release of irSP from the spinal cord were SP-containing sites and that such sites exhibited neuronal activation by CoAO, both Fos immunohistochemistry
Intact dorsal roots (see Fig. 1).

Rats undergoing transection of the dorsal roots from T2–VI around the central canal, lamina X (Fig. 6C), and laminae I–III (Fig. 6B), around the central canal, lamina X (Fig. 6C), and laminae VI–VII (Fig. 6D).

The experiments involving CoAO were repeated in a separate group of rats that underwent dR of spinal segments T2–T5. Rats undergoing transection of the dorsal roots from T2–T5 overall had a lower resting BP and higher HR than rats with intact dorsal roots (see Fig. 1F and Table 1). However, rhizotomy did not attenuate the depressor response to CoAO because MBP decreased by 14 mmHg in group 2 rats (CoAO) and by 15 mmHg in group 3 (CoAoD) rats (see Table 1). Except for a residual release of irSP from the most superficial dorsal laminae, dorsal rhizotomy of the upper thoracic segments eliminated the background release of irSP from spinal laminae III–VII (Fig. 7A). Moreover, after dR, rest and CoAO probes showed similar binding profiles (Fig. 7B), indicating minimal alteration in SP release profile by this intervention. This is in marked contrast to the significant difference between the average gray levels of the CoAO probes and the CoAoD probes (Fig. 8). The lamina sites responsible for the release of endogenous irSP picked up by the antibody-coated microprobes are illustrated in Fig. 9. Shown in this figure is a tissue section from a T3 spinal segment that was stained for neuronal activation using Fos. Microprobes were inserted into the left side of the spinal cord, 0.4–0.5 mm lateral to the midline. Superimposed just to the left of this (to allow visualization of the Fos-immunostained neurons, i.e., dark spots in the gray mater) is a pseudocolor image of a microprobe during CoAO. Note that the segment of the probe residing in the spinal cord has less intense binding of radiolabeled SP than the outside (image less intense; see MATERIALS AND METHODS for corresponding gray level intensity). The lamina responsible for the SP release are obtained by aligning the Student’s T graph (from Fig. 5B) over the left side of the spinal segment and identifying the sites where there was a significant difference in the gray levels between the rest and CoAO probes (the T-graph is positioned over to the right for illustrative purposes to visualize the active neuronal sites). Note also that sites of release are also sites of activation (as determined by Fos) and SP-containing sites (as seen in Fig. 6). The sites of release correspond to the active neuronal sites (as determined by Fos) and SP-containing sites (as seen in Fig. 6). The sites of release correspond to the active neuronal sites (as determined by Fos) and SP-containing sites (as seen in Fig. 6). The sites of release correspond to the active neuronal sites (as determined by Fos) and SP-containing sites (as seen in Fig. 6). The sites of release correspond to the active neuronal sites (as determined by Fos) and SP-containing sites (as seen in Fig. 6).

DISCUSSION

SP is found in abundance in the spinal cord, in endings of primary afferent nerves, which terminate mainly in laminae I, II, and V (29), and is known to have a crucial role in transmission of nociceptive inputs (5, 10, 12, 31). The data
reported here show that differential activation of left ventricular nociceptive afferent fibers by transient and focal ischemia causes the increase release of SP from the superficial dorsal laminae I and II, down to and including the deeper lamina, III–VII. This increased release was associated with the activation of the CNAN, because this profile was maintained only for time when the coronary occlusion was being applied. These data suggest that the SP released in spinal cord dorsal horn sites is a principal neuropeptide mediator associated with signal transmission of myocardial ischemia and nociception. Conversely, diffuse chemical activation of epicardial cardiac afferents with a mixture of algogenic substances (bradykinin, 5-hydroxytryptamine, adenosine, and PGE2) did not alter the pattern of SP release in these same areas of the T4 spinal segment. These data suggest that differential activation of cardiac sensory neurites results in differential and discrete release of neuromediators from their associated spinal cord projections.

Virtually no information exists about the release of neuropeptides in the spinal cord in response to noxious stimuli applied to cardiac afferent neurons. Two experimental approaches were used to stimulate CNAN. The first, intrapericardial IES infusion, has been used as a model to simulate the myocardial extracellular changes in algogenic substances that are known to stimulate CNAN (14, 25), whereas the second, CoAO, has been used to reproduce the ischemic stimulus that precipitates the conditions leading to angina (25). Arguments can be made that applying these techniques in an anesthetized animal prohibits observation of behavioral responses that would demonstrate pseudoadfective or pain-like reactions, but, as pointed out by Euchner-Wamser et al. (13) and Meller and Gebhart (25), there is strong experimental evidence that both models activate cardiac afferent neurons and that both produce the behavioral and electrophysiological effects consistent with angina. Cardiac nociceptive afferent neurons, with somata in the dorsal root ganglion, have small diameter axons (Aδ- and c-fibers) that terminate mainly on laminae I, V, VII, and X (14, 23). Their sensory neurites are multimodal transducing mechanical and chemical stimuli (18, 19, 33). Inputs from subpopulations of cardiac afferents subserve normal cardiovascular regulation (19), as well as nociception when excessively activated (19). Sensory afferent neurons of this category are known to contain SP (29); their tachykinin receptors are located predominately in laminae I, II, and X (32), sites where CNAN terminate. Thus the anatomic evidence detailing the circuitry of CNAN and location of SP and its receptors in the spinal cord support the suggestion that SP can serve a role as a neuromodulator for cardiac ischemic signaling.

In the present study, we found a basal release of SP in the thoracic spinal cord when innervation between the intrathoracic aspects of the cardiac nervous system and spinal cord were intact. Other studies (35, 36) have reported a basal release of SP at various points in the CNS using this and other techniques. In the current study, this basal release remains fairly stable over a 3- to 4-h period, well after the completion of this surgical preparation. This suggested the background release is not a function of the surgery, but reflective of visceral input, descending input from higher centers (17), and/or intersegmental interactions. Dorsal rhizotomy mitigated the basal SP release, except at the most superficial dorsal root segments and prevented the subsequent SP release evoked by transient cardiac occlusion. The basal release of SP from superficial laminae after rhizotomy may reflect a residual release of anterior descending coronary artery increased release of irSP from the superficial dorsal laminae I and II, down to and including the deeper lamina, III–VII. This increased release was associated with the activation of the CNAN, because this profile was maintained only for time when the coronary occlusion was being applied. These data suggest that the SP released in spinal cord dorsal horn sites is a principal neuropeptide mediator associated with signal transmission of myocardial ischemia and nociception. Conversely, diffuse chemical activation of epicardial cardiac afferents with a mixture of algogenic substances (bradykinin, 5-hydroxytryptamine, adenosine, and PGE2) did not alter the pattern of SP release in these same areas of the T4 spinal segment. These data suggest that differential activation of cardiac sensory neurites results in differential and discrete release of neuromediators from their associated spinal cord projections.

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In the present study, we found a basal release of SP in the thoracic spinal cord when innervation between the intrathoracic aspects of the cardiac nervous system and spinal cord were intact. Other studies (35, 36) have reported a basal release of SP at various points in the CNS using this and other techniques. In the current study, this basal release remains fairly stable over a 3- to 4-h period, well after the completion of this surgical preparation. This suggested the background release is not a function of the surgery, but reflective of visceral input, descending input from higher centers (17), and/or intersegmental interactions. Dorsal rhizotomy mitigated the basal SP release, except at the most superficial dorsal root segments and prevented the subsequent SP release evoked by transient cardiac occlusion. The basal release of SP from superficial laminae after rhizotomy may reflect a residual release of anterior descending coronary artery increased release of irSP from the superficial dorsal laminae I and II, down to and including the deeper lamina, III–VII. This increased release was associated with the activation of the CNAN, because this profile was maintained only for time when the coronary occlusion was being applied. These data suggest that the SP released in spinal cord dorsal horn sites is a principal neuropeptide mediator associated with signal transmission of myocardial ischemia and nociception. Conversely, diffuse chemical activation of epicardial cardiac afferents with a mixture of algogenic substances (bradykinin, 5-hydroxytryptamine, adenosine, and PGE2) did not alter the pattern of SP release in these same areas of the T4 spinal segment. These data suggest that differential activation of cardiac sensory neurites results in differential and discrete release of neuromediators from their associated spinal cord projections.

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Fig. 5. Image analysis graphs of SP antibody-coated microprobes during CoAO. A: comparison of the binding patterns of rest probes to in vitro probes; B: rest to CoAO probes; C: rest and recovery probes, and D: CoAO and recovery probes. Details of graphs described in Fig. 3.

Fig. 6. Sites of neuronal activation and SP location in rat thoracic spinal cord at T4. A: cross-section of rat spinal cord transilluminated showing Fos-positive neurons in response to CoAO (black spots) viewed through a Nikon zoom lens. B–D: sections of spinal cord from same animal with SP labeled with FITC showing the location of SP-containing sites (B), dorsal horns (C) around the central canal, and ventral horns (D) as viewed through a Nikon confocal microscope.
neuropeptide as a result of the transection of dorsal root fibers and the removal of regulated processes. It might also reflect the interactions of residual intersegmental interconnections. The differences in the profiles of the microprobes during the control CoAO, with dorsal roots intact, compared with CoAO after rhizotomy (Fig. 8), demonstrate that SP is released from laminae I–VII in response to a cardiac ischemic stress, primarily as the result of cardiac neural afferent inputs. This profile does not preclude the possibility that descending projections may contribute to and modulate spinal cord processing of that sympathetic afferent input. In this regard, Foreman and co-workers (15) have proposed a vagal afferent neuron-mediated modulation of thoracic cord reflex function utilizing information processing circuits localized within the upper cervical cord.

Cardiac sensory afferent neurons transduce chemical and mechanical stimuli from the heart. Electrophysiological studies have previously shown that intrapericardial infusion of algogenic substances as done herein excites CNAN and these, in turn, excite neurons in the dorsal horn of the spinal cord (7, 14, 25, 28). Chemical stimulation of cardiac afferents causes an increase in the discharge rate of subpopulations of spinal neurons as long as their receptors are exposed to the chemical stimuli (3, 4, 7, 18, 28, 39). Yet activation of CNAN by the IES solution did not detectably alter the pattern of SP release at the T4 level, at least when evaluated 0.5 mm lateral to the midline to a depth of 2 mm below the dorsal surface. First, the reasons for the divergence of the SP release response from that noted with coronary artery occlusion may be multifactor and include the position of the probes, the intensity of afferent fiber stimulation, the diffusivity of the excitatory signal, and the

![Image](https://example.com/image1.png)

**Fig. 7.** Image analysis graphs of SP antibody-coated microprobes during CoAO after dorsal rhizotomy (dR) of spinal segments T2–T5. A: comparison of the binding patterns of rest probes following rhizotomy to in vitro probes. B: rest probes to CoAO probes; $n$, number of probes in each group. See Fig. 3 for details of graphs.

![Image](https://example.com/image2.png)

**Fig. 8.** Influence of rhizotomy on SP release. The histograms represent the differences in the mean area ± SE under the peaks of irSP release from the spinal cord in laminae I–VI. Microprobes were inserted into T4 in rats with intact dorsal afferents during CoAO or during CoAO in rats after dorsal afferent rhizotomy (CoAO+dR) compared with rest. Numbers of probes shown in parenthesis, GS, gray scale. The mean areas were compared using Student’s $t$-test for unpaired data. *$P < 0.05$.

![Image](https://example.com/image3.png)

**Fig. 9.** Sites of release of SP. Cross-section of T4 spinal segment stained with 3',3'-diaminobenzidine-Fos-positive neurons activated in response to CoAO and superimposed $t$-test (from Fig. 5B) illustrating sites along microprobes, where gray levels of the CoAO probes were significantly lower than the rest probes, indicating release of endogenous SP. Pseudocolor image of SP antibody-coated microprobe superimposed on the left side of the section illustrating the lower intensity binding of radiolabeled SP, corresponding to the sites of release of endogenous SP.
diversity of the afferent response or neurotransmitter differences. Previously, with the use of Fos-immunohistochemistry, IES infusion caused activation of more cells in the lateral portion of the dorsal horns in the thoracic spinal cord than in the medial portion (author’s unpublished observations). Because the microprobe can detect differences in binding with a resolution of 100 μm, it is possible that sites of release of SP from IES-activated CNAN were outside this range. However, when probes were placed at the more lateral position (0.7 mm from the midline), IES infusion failed to induce an increased release of SP above the resting levels. This finding would appear to preclude this possibility. Second, IES infusion may cause the microprobe can detect differences in binding with a fundamental to the antianginal effects of spinal cord stimulation.

Heterogeneous activation of cardiac afferent neurons, occurring with focal cardiac ischemia, represents an optimum stimulus for the cardiac neuronal hierarchy. This originating stimulus at the myocardial level most likely is due to not only chemoactivation of the CNAN but also some mechanoactivation of these fibers. Therapies directed at minimizing sensory receptor adaptation, which typically would result in a reduction in firing rates. Third, IES infusion activates only the most superficial CNAN and in a diffuse and homogenous manner. In other works, the pattern of afferent neuronal activation (discrete vs. diffuse, superficial vs. deep, etc.) may significantly impact on the degree of activation spinal cord elements of the cardiac nervous system. Fourth, chemical activation of cardiac sensory afferents can elicit directionally different responses, some increasing and other decreasing their activity (19). As such, there could be a net cancellation of response in spinal cord processing centers and as such a reduced release of SP. Finally, different populations of cardiac afferents, responding differentially to cardiac stress, may utilize different neurotransmitters. This resolution of these various possibilities awaits further exploration.

**Prospectus.** Heterogeneous activation of cardiac afferent neurons, occurring with focal cardiac ischemia, represents an optimum stimulus for the cardiac neuronal hierarchy. This originating stimulus at the myocardial level most likely is due to not only chemoactivation of the CNAN but also some mechanoactivation of these fibers. Therapies directed at minimizing the disparities in the chemical/mechanical milieu in the progression of ischemic heart disease should be reflected in lesser activation of the cardiac neuronal hierarchy and as such reduce angina. Recent evidence has suggested that discrete electrical activation of dorsal segments of T1–T3 elements of the spinal cord restores such a balance in the intrapericardial aspects of the cardiac nervous system (15) and as such may be fundamental to the antianginal effects of spinal cord stimulation (1). Further investigation is needed to determine whether such therapy mitigates SP release at the spinal cord.

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