Functional role of peripheral opioid receptors in the regulation of cardiac spinal afferent nerve activity during myocardial ischemia

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Fu LW, Longhurst JC. Functional role of peripheral opioid receptors in the regulation of cardiac spinal afferent nerve activity during myocardial ischemia. Am J Physiol Heart Circ Physiol 305: H76–H85, 2013. First published May 3, 2013; doi:10.1152/ajpheart.00091.2013. —Thickly myelinated Aδ-fiber and unmyelinated C-fiber cardiac sympathetic (spinal) sensory nerve fibers are activated during myocardial ischemia to transmit the sensation of angina pectoris. Although recent observations showed that myocardial ischemia increases the concentrations of opioid peptides and that the stimulation of peripheral opioid receptors inhibits chemically induced visceral and somatic nociception, the role of opioids in cardiac spinal afferent signaling during myocardial ischemia has not been studied. The present study tested the hypothesis that peripheral opioid receptors modulate cardiac spinal afferent nerve activity during myocardial ischemia by suppressing the responses of cardiac afferent nerve to ischemic mediators like bradykinin and extracellular ATP. The nerve activity of single unit cardiac afferents was recorded from the left sympathetic chain (T2–T3) in anesthetized cats. Forty-three ischemically sensitive afferent nerves (conduction velocity: 0.32–3.90 m/s) with receptive fields in the left and right ventricles were identified. The responses of these afferent nerves to ischemia or ischemic mediators were further studied in the following protocols. First, epicardial administration of naloxone (8 μmol), a nonselective opioid receptor antagonist, enhanced the responses of eight cardiac afferent nerves to recurrent myocardial ischemia by 62%, whereas epicardial application of vehicle (PBS) did not alter the responses of seven other cardiac afferent nerves to ischemia. Second, naloxone applied to the epicardial surface facilitated the responses of seven cardiac afferent nerves to epicardial ATP by 76%. Third, administration of naloxone enhanced the responses of seven other afferent nerves to bradykinin by 85%. In contrast, in the absence of naloxone, cardiac afferent nerves consistently responded to repeated application of ATP (n = 7) or bradykinin (n = 7). These data suggest that peripheral opioid peptides suppress the responses of cardiac sympathetic afferent nerves to myocardial ischemia and ischemic mediators like ATP and bradykinin.

sympathetic afferent nerves; opioid receptors; myocardial ischemia; naloxone

Activation of thickly myelinated Aδ-fiber and unmyelinated C-fiber cardiac sympathetic (spinal) afferent nerves during myocardial ischemia is responsible for the transmission of information from the heart to the brain that ultimately elicits the perception of cardiac pain and evokes excitatory cardiovascular reflex responses (19, 22, 39). For many years, attention has been focused on characterizing the excitatory effects elicited by a number of ischemic mediators on cardiac spinal afferent nerve activity during myocardial ischemia. In contrast, inhibitory influences of ischemic mediators on cardiac spinal afferent nerve excitability have not been studied. For example, we and others (3, 15, 17–19, 21, 64) have demonstrated that endogenously produced ischemic metabolites, including endothelin, protons, extracellular ATP, thromboxane A2, serotonin (5-HT), histamine, ROS, and bradykinin (BK), excite cardiac spinal afferent nerves during ischemia and receptor-ffusin in an interactive and multifactorial fashion. Some mediators, like ATP and 5-HT, are specific for ischemically sensitive afferent nerves (15, 20), whereas others, like BK, ROS, and histamine, are nonspecific, as they stimulate both ischemically sensitive and insensitive afferent nerve endings (16, 28, 61).

The endogenous opioid system in the brain and spinal cord, which function as atypical inhibitory neurotransmitters or neuromodulators, have been extensively studied (34, 35, 62, 63, 69). However, the functional effects of this system on peripheral sensory nerve activity have been investigated less extensively. Several sources of evidence suggest that peripheral opioids may be crucial in the regulation of cardiac spinal afferent nerve activity during myocardial ischemia. For instance, cardiac myocytes, sympathetic nerves, and leukocytes can synthesize and store opioid peptides as well as their precursors (38, 41, 44, 67). Leukocytes that accumulate in the area of coronary arterial plaques release large quantities of opioids when they are activated during plaque disruption (36, 38, 46). Clinical studies (5, 7, 27) have documented increased concentrations of coronary opioid peptides in patients with unstable angina pectoris or undergoing coronary angioplasty. Hence, myocardial ischemia leads to the production and release of opioid peptides that have the potential to contribute to the regulation of cardiac spinal afferent nerve activity during ischemia.

Opioids exert their physiological actions mainly through stimulation of opioid μ-, δ-, and κ-receptors (31, 59). Anatomical studies (13, 24, 42, 59) have found all three opioid receptors on sensory neurons in the dorsal root ganglia (DRG). However, the responses of peripheral somatic and visceral sensory nerves to exogenous opioids are controversial (2, 9, 10, 25, 30, 52). Some investigators (25, 55) have observed that opioid receptor agonists markedly excite rat mesenteric afferent fibers and mouse sensory DRG neurons and that these opioid actions are eliminated by blockade of opioid receptors. In contrast, Wenk and colleagues (68) demonstrated that opioids inhibit the majority of somatic Aδ- and C-fiber nociceptors innervating inflamed skin. Opioid μ- and κ-receptor agonists also suppress ferret esophageal vagal afferent mechanosensitivity (49). However, no studies have examined the role of endogenous opioids in the regulation of cardiac sensory nerve
activity. Because opioid action in the central nervous system is inhibitory (34, 62, 63), we speculated that endogenously produced opioids likely modulate cardiac spinal afferent nerve activity during myocardial ischemia.

The ischemic mediators BK and ATP contribute to the excitation of cardiac spinal afferent nerves during myocardial ischemia (20, 61). BK B$_2$ (BK$_2$) receptors are G protein-coupled receptors (GPCRs) that colocalize with opioid δ-receptors in trigeminal ganglion neurons (4, 32). BK activates cardiac spinal and somatic sensory fibers through excitation of BK$_2$ GPCRs linked to excitatory G$_{4}$ protein (32, 61, 70). Opioid receptors are similarly coupled to the GPCR family. However, they are linked to inhibitory G$_{1}$ and G$_{2}$ proteins, which decrease cAMP and inhibit voltage-dependent Ca$^{2+}$ channels, resulting in reduced nerve activity (4, 31, 33, 66).

The shared parts of the GPCR signaling pathways for opioids, BK, and ATP suggest that they have the potential to interact when released concomitantly during myocardial ischemia. Additionally, opioids have the potential to modulate cardiac afferent nerve responses to extracellular ATP because ATP excites peripheral sensory nerve fibers, in part, through their action on P2X receptor-linked ligand-gated ion channels (6, 20), which are inhibited when opioid µ-receptor agonists are applied to sensory nerves (59).

The aim of the present study, therefore, was to determine the functional role of endogenously produced opioid peptides in the modulation of cardiac spinal afferent nerve activity during ischemia. We evaluated the influence of opioids on the responses of ischämically sensitive afferent nerves to BK and ATP as well as on cardiac afferent nerve activity during myocardial ischemia. We recorded single unit nerve activity to test the hypothesis that endogenous opioids modulate the activity of cardiac sympathetic afferent nerves during myocardial ischemia by suppressing the responses of these sensory nerve fibers to the ischemic mediators BK and extracellular ATP. A portion of these data have been published as a preliminary report (14).

METHODS

Surgical Preparation

Forty-three adult cats of either sex (2.32–4.15 kg) were anesthetized by an intramuscular injection of ketamine (20–30 mg/kg, Phoenix Scientific, St. Joseph, MO) followed by an intravenous injection of α-chloralose (40–50 mg/kg) through the femoral vein. The trachea of each animal was intubated, and respiration was maintained artificially (Harvard pump model 661, South Natick, MA). Cats were ventilated by air supplemented with 100% O$_2$ through the respirator. A femoral vein and artery were cannulated for the administration of drugs and fluids and the measurement of blood pressure. A pressure transducer (Statham P 23 ID, Gould) was connected to the femoral arterial catheter for measurements of arterial blood pressure. Arterial blood gases were assayed frequently with a blood gas analyzer (Radiometer ABL-5, Copenhagen, Denmark) and were maintained within physiological limits (P$_{O_2}$ > 100 mmHg, P$_{CO_2}$ = 28–35 mmHg, pH 7.35–7.45) by adjusting the respirator rate or tidal volume or by the intravenously administration of 2–3 ml of 1 M of NaHCO$_3$ [8.4% (wt/vol)]. Additional injections of α-chloralose (5–10 mg/kg iv) were given as necessary to maintain an adequate depth of anesthesia, which was assessed by observing the absence of a conjunctival reflex. Body temperature was monitored with a rectal thermometer and maintained at 36–38°C with a circulating water heating pad and a heat lamp. Animals were euthanized at the end of each experiment by administration of a solution of saturated KCl into the femoral vein under deep anesthesia, ensured by administration of an additional dose of α-chloralose (50 mg/kg). The surgical and experimental protocols used in this study were approved by the Animal Use and Care Committee of the University of California (Irvine, CA). The experiments conformed with American Physiological Society’s “Guiding Principles in the Care and Use of Animals.”

Cardiac Spinal Afferent Nerve Recordings

Single unit activity of cardiac afferent nerves was recorded as previously described (15, 16). In brief, a midline sternotomy was performed, and the first to seventh left ribs and left lung were removed. Small nerve filaments were dissected gently from the chain and rami communicates, covered with warm mineral oil, between T$_2$ and T$_5$ under a microscope (Zeiss). The rostral ends were placed across a recording electrode that was attached to a high-impedance probe (model HIP511, Grass Instruments, Quincy, MA). Action potentials were amplified (×50,000) and bandpass filtered (100–3,000 Hz) through an alternating current amplifier (model P511, Preamp + Grass) and processed through an audioamplifier (AMSB, Audiomonitor, Grass) and an oscilloscope (model 2201, Tektronix, Beavertown, OR). Nerve activity and blood pressure signals were recorded on a Pentium computer using data acquisition and analysis software (Spike2), which sampled these signals at 10,000 Hz through an analog-to-digital converter (CED micro 1401 mkII, Cambridge, UK) for online and offline quantitative analysis. Action potentials in the neurogram were analyzed both visually and with the Spike2 program, which used waveform recognition algorithms to allow accurate detection of similar wave shapes and heights and hence the precise calculation of discharge frequency for each afferent nerve.

The location of the afferent nerve ending was identified by placing a stimulating electrode directly on the surface of the myocardium to evoke the afferent nerve’s action potential, as previously described (15, 18). Locations of all afferent nerve endings were confirmed through mechanical stimulation of the heart by gently probing the epicardial surface with a cotton swab and constricting the thoracic aorta as well as by chemical stimulation with epicardial application of BK (2–3 µg). The conduction velocity (CV) of each afferent nerve fiber was calculated by dividing conduction distance by conduction time. Conduction time was determined by measuring the time interval from electrical stimulation to the evoked afferent nerve’s action potential. These evoked traces were overlaid multiple times at the same time point as they were displayed on the oscilloscope and computer monitor. Conduction distance was estimated by measuring the length of a wet thread along the supposed path through the stellate ganglion from the receptive field to the recording electrode. Unmyelinated C-fiber and thinly myelinated Aδ-fiber afferent nerves were classified as those with CVs of <2.5 and 2.5–30 m/s, respectively. In the present study, each afferent nerve had a single receptive field that could be located precisely in the left or right ventricles. Myocardial ischemia was induced by complete occlusion of the appropriate coronary artery supplying the regional receptive field of the cardiac afferent nerve. Ischemia was confirmed by observing a regional change in the color of the myocardium (50). Afferent nerves were considered to be ischemically sensitive if their discharge activity during 3–5 min of myocardial ischemia increased at least 50% above baseline. We found that 76% of the afferent nerves in this study were ischemically sensitive. To determine whether an afferent nerve was chemosensitive, BK (2–3 µg) was applied to the surface of the ventricle with a pledget, and the afferent nerve response was recorded. Afferent nerves were classified mechanosensitive if their activity increased at least 50% above baseline during aortic constriction, which raised systolic blood pressure to 170–190 mmHg for 15 s and is associated with increases in both cardiac pressure and volume (15, 20). In the present study, only one ischemically sensitive cardiac afferent nerve was studied in each animal.
Experimental Protocols

Two groups of animals were studied for each protocol, one group for the active intervention and another group serving as a time control.

Influence of opioid receptor blockade on cardiac afferent nerve responses to ischemia. To examine whether blockade of opioid receptors with the specific antagonist naloxone (48, 68) altered afferent nerve responses to myocardial ischemia, we measured afferent nerve responses (imp/s) to repeated ischemia before and after the epicardial application of naloxone (8 μmol) in eight animals. In a pilot study, we examined the effects of 4 and 8 μmol naloxone on the cardiac afferent nerve response to ischemia. We observed that 4 μmol naloxone facilitated the responses to ischemia in two of the three afferent nerves, whereas 8 μmol naloxone enhanced the responses of each of the three other afferent nerves. Thus, for the remainder of this protocol, we used 8 μmol naloxone. Previous studies (48, 68) also have demonstrated that at this dose, naloxone completely eliminates.

Fig. 1. Diagrammatic graph showing the location of the receptive fields of 43 ischemically sensitive cardiac afferent nerves on the epicardial surface of the left (n = 41) and right (n = 2) ventricles. The following protocols included receptive fields of cardiac afferent nerves in the study: ischemia + naloxone (●; n = 8), repeat ischemia (●; n = 7), ATP + naloxone (●; n = 7), repeat ATP (●; n = 7), bradykinin (BK) + naloxone (●; n = 7), and repeat BK (●; n = 7).

Fig. 2. Effect of opioid receptor blockade with the specific antagonist naloxone on ischemia-mediated responses of a left ventricular afferent nerve [anterior wall, conduction velocity (CV) = 0.45 m/s]. Before blockade (A), the afferent nerve response increased from 0.23 to 3.83 impulses/s (imp/s) during ischemia. After blockade (B), the C-fiber response to repeat ischemia was enhanced by 39%. Neurograms 1–4 are representative tracings of afferent nerve responses at the times shown by the arrows in the histograms.

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the response of somatic sensory nerve to opioids by antagonizing opioid μ-, δ-, and κ-receptors. After the receptive field of an afferent nerve was located in the heart, the response of the afferent nerve to brief (5 min) ischemia was evaluated. If the afferent nerve did not respond to an initial period of ischemia, we relinquished it and searched for another afferent that was responsive. If the afferent nerve responded to ischemia, naloxone was applied to the receptive field on the surface of the heart with 1- to 1.5-cm² filter paper for 5 min, and ischemia was repeated immediately thereafter for the same length of occlusion 30 min after the initial period of ischemia. Naloxone (Sigma-Aldrich, St. Louis, MO) was dissolved in vehicle (PBS, pH 7.35) to a concentration of 80 mM. The pH of the solution was adjusted with NaHCO₃ [8.4% (wt/vol)] to a final value of 7.35. The naloxone solution was prepared weekly and stored at −20°C.

To evaluate the reproducibility of afferent nerve responses to ischemia, seven additional ischemically sensitive afferent nerves in seven animals were studied as time controls. Each animal in this group was treated identically with the exception that an epicardial application of vehicle (PBS, 0.1 ml) was used in place of naloxone.

**Effect of opioid receptor blockade on afferent nerve responses to ATP.** This protocol consisted of two groups of afferent nerves used to determine the influence of blockade of opioid receptors with naloxone on afferent nerve responses to extracellular ATP. The response to ischemia was evaluated after the receptive field of an afferent nerve was located on the heart. If the afferent nerve was ischemically sensitive, we recorded the afferent nerve response to epicardial application of ATP (2 μmol). Responses of seven afferent nerves to repeated epicardial ATP were evaluated 5 min after the epicardial application of naloxone (8 μmol) and 30 min after the initial application of ATP. ATP (Sigma-Aldrich) was dissolved in PBS to a concentration of 20 mM. ATP solutions were prepared weekly and stored at −20°C.

To differentiate between variations in afferent nerve responses to ATP and time-related effects, seven additional afferent nerves were studied as time controls. After identification, each ischemically sensitive unit was treated identically to the intervention group except that PBS (0.1 ml) was used in place of naloxone.

**Influence of opioid receptor blockade on afferent nerve responses to BK.** We evaluated the influence of naloxone on afferent nerve responses to BK in two groups of afferent nerves. In the first group, after we identified the location of the receptive field in the ventricle, afferent nerve activity was measured during 3–5 min of myocardial ischemia. Afferent responses to epicardial BK (1 μg) were evaluated in seven ischemically sensitive afferent nerves. The discharge activity in response to repeated BK was evaluated 5 min after the epicardial application of naloxone (8 μmol) and 30 min after the initial BK application. BK (Sigma-Aldrich) was dissolved in PBS to a concentration of 10 μg/ml. BK solutions were prepared weekly and stored at −20°C.

To determine the reproducibility of afferent nerve responses to BK, a second group of seven afferents was studied as time controls. After identification, each ischemically sensitive unit was treated identically to the intervention group except that vehicle (PBS, 0.1 ml) was used in place of naloxone.

**Data Analysis**

The discharge activity of cardiac spinal afferent nerves was expressed in impulses per second and averaged during the 3- to 5-min preischemia period and 5 min of ischemia. We measured the responses of cardiac afferents to ATP, BK, and naloxone by averaging the discharge rates of the afferent nerves during the entire period of response, defined as the time during which sustained activity exceeded baseline activity by 20%. During drug application, sampling periods varied between 70 to 400 s, depending on the responses of the afferents to the drug. Five-minute sampling periods were used to measure afferent nerve activity during myocardial ischemia. Baseline activity was determined over the 3- to 5-min period immediately preceding ischemia. We also evaluated the total response of afferent nerves to ischemia or chemical stimulation by assessing the number of spikes that occurred during the entire response.

Data are expressed as means ± SE. A Shapiro-Wilk test was used to determine if the data were distributed normally. Normally distributed data in all protocols were compared with either a Student’s paired t-test for paired data or a one-way repeated-measures ANOVA followed by the Holm-Sidak’s post hoc test. Non-normally distributed data in all protocols were compared with Friedman repeated-measures ANOVA on ranks followed by Tukey’s post hoc test. All statistical calculations were performed with SigmaStat software (Jandel Scientific, San Rafael, CA). Values were considered to be significantly different when *P < 0.05.

**RESULTS**

**Profile of Cardiac Afferent Nerves**

Forty-three ischemically sensitive cardiac afferent nerves in forty-three animals were evaluated in the present study. Approximately 31% of the endings of these nerves were mechano-sensitive (responsive to aortic constriction), and all were chemosensitive (responsive to BK). The endings of 41 afferents were located in the anterior (n = 17) or posterior (n = 24) wall of the left ventricle, whereas 2 endings were located in the anterior (n = 1) or posterior (n = 1) wall of the right ventricle (Fig. 1). All afferent nerves had a single receptive field. CVs varied between 70 to 400 s, depending on the responses of the afferents to the drug.
ranged between 0.32 and 3.9 m/s. Ninety-three percent (40 fibers) of the afferents were classified as C-fibers. The remaining units (3 afferent nerves) were Aδ-fibers. There were insufficient data to test for an association between CV and the responsiveness of the fibers to mechanical or chemical stimulation or ischemia.

**Effect of Blockade of Opioid Receptors on Responses of Cardiac Afferent Nerves to Ischemia**

Representative discharge activities of a cardiac C-fiber afferent nerve that responded to myocardial ischemia in the absence and presence of naloxone are shown in Fig. 2. Ischemia increased the activity of this nerve from 0.23 to 3.83 impulses/s (Fig. 2A). Opioid receptor blockade with the epicardial application of naloxone (8 μmol) enhanced ischemia-induced increases in cardiac afferent nerve activity by 39% when their activity was averaged over a 5-min period (3.83 to 5.31 impulses/s; Fig. 2B).

The responses of two groups of cardiac spinal afferent nerves to brief myocardial ischemia are shown in Fig. 3 and Table 1. In the first group, 5 min of ischemia significantly increased the discharge activity of eight afferents (CV = 0.71 ± 0.17 m/s) from 0.92 ± 0.17 to 2.79 ± 0.39 impulses/s (Fig. 3A). The ischemia-evoked increase in afferent nerve activity was 1.78 ± 0.36 impulses/s after the subtraction of baseline activity. Ischemia did not alter mean arterial blood pressure (MAP; 98 ± 7 vs. 94 ± 9 mmHg, before vs. during ischemia, P > 0.05). However, after antagonism of opioid receptors with naloxone (8 μmol), the ischemia-induced increase in afferent nerve activity was facilitated significantly by 62% (1.78 ± 0.36 vs. 2.89 ± 0.55 impulses/s, initial vs. repeated ischemia, P < 0.05; Fig. 3A). The total response of afferent nerves to ischemia over the entire duration was enhanced in the presence of naloxone (Table 1). We did not observe any afferents that became active during repeat ischemia after naloxone. MAP was unchanged during repeat ischemia (99 ± 7 vs. 93 ± 8 mmHg, before vs. during ischemia, P > 0.05). In the second group of seven afferent nerves (one Aδ-fiber, CV = 3.9 m/s, and six C-fibers, CV = 0.47 ± 0.05 m/s), the vehicle did not affect the total or peak responses to

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**Table 1. Duration and total responses of cardiac afferent nerves to ischemia, ATP, and BK before and after naloxone**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Duration, s</th>
<th>Total Responses, impulses</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
<td>Repeat</td>
</tr>
<tr>
<td>Ischemia + naloxone</td>
<td>8</td>
<td>300 ± 0</td>
<td>300 ± 0</td>
</tr>
<tr>
<td>Ischemia + vehicle</td>
<td>7</td>
<td>300 ± 0</td>
<td>300 ± 0</td>
</tr>
<tr>
<td>ATP + naloxone</td>
<td>7</td>
<td>88 ± 8</td>
<td>177 ± 21*</td>
</tr>
<tr>
<td>ATP + vehicle</td>
<td>7</td>
<td>86 ± 7</td>
<td>82 ± 7</td>
</tr>
<tr>
<td>BK + naloxone</td>
<td>7</td>
<td>82 ± 6</td>
<td>208 ± 38*</td>
</tr>
<tr>
<td>BK + vehicle</td>
<td>7</td>
<td>78 ± 7</td>
<td>81 ± 8</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of nerves. Ischemia + naloxone, ischemia stimulation before and after administration of naloxone; the same format is used with other mediator combinations. BK, bradykinin. *P < 0.05 compared with the initial stimulus.

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Fig. 4. Effect of opioid receptor blockade with naloxone on responses of left ventricular afferent nerves (posterior wall, CV = 0.63 m/s) to epicardial application of ATP. Before blockade (A), the cardiac afferent response to epicardial ATP increased from 0.38 to 2.0 imp/s. After blockade (B), the response to ATP was facilitated (0.46 to 3.72 imp/s). Neurograms 1 and 2 are representative tracings of afferent nerve responses at the times shown by lines in the histograms.
ischemia (Fig. 3B and Table 1). Naloxone alone did not change the baseline activity of these afferent nerves (0.83 ± 0.21 to 0.87 ± 0.25 impulses/s, \(P > 0.05\)). The locations of the 15 afferent nerve endings studied in this protocol are shown in Fig. 1.

**Influence of Opioid Receptor Blockade on Cardiac Afferent Nerve Responses to ATP**

An example of an ischemically sensitive cardiac afferent nerve response to epicardial ATP before and after the administration of naloxone is shown in Fig. 4. ATP increased the discharge activity of this nerve from 0.38 to 2.0 impulses/s (Fig. 4A). Naloxone enhanced the ATP-elicited increase in this afferent nerve activity by 86% (2.0 to 3.72 impulses/s; Fig. 4B).

The responses of two groups of cardiac afferent nerves to 2 \(\mu\)mol ATP are shown in Fig. 5 and Table 1. ATP significantly increased the discharge activity of seven afferents (one A\(\delta\)-fiber, \(CV = 3.31\) m/s, and six C-fibers, \(CV = 0.53 \pm 0.19\) m/s) from 0.76 ± 0.07 to 2.1 ± 0.11 impulses/s in the first group (Fig. 5A). The increase in the afferent response to repeated epicardial ATP was enhanced by 76% after opioid receptor blockade with 8 \(\mu\)mol epicardial naloxone (1.34 ± 0.23 vs. 2.36 ± 0.35 impulses/s, initial vs. repeated ATP, \(P < 0.05\)). The total response and response duration of these afferent nerves to ATP likewise were facilitated by opioid receptor antagonism (Table 1). The activity of these afferent nerves was unchanged by naloxone in the absence of ATP. MAP was unchanged during the initial and repeat ATP stimulation (initial: 94 ± 8 vs. 99 ± 10 mmHg and repeat: 93 ± 7 vs. 96 ± 9 mmHg, before vs. after ATP, both \(P > 0.05\)). Seven additional C-fiber afferent nerves (\(CV = 0.63 \pm 0.12\) m/s) in the second group responded consistently to ATP after the administration of vehicle (PBS; Fig. 5B). The locations of the 14 afferent nerve endings are shown in Fig. 1.

**Endogenous Opioid Modulation of Cardiac Afferent Nerve Responses to BK**

A histogram of the neural activity showing the influence of opioid receptor blockade with naloxone on the response of an ischemically sensitive cardiac C-fiber afferent nerve to BK is shown in Fig. 6A. In the absence of naloxone, epicardial application of BK (1 \(\mu\)g) increased the activity of this afferent nerve from 0.62 to 1.73 impulses/s. Opioid receptor blockade with naloxone enhanced the response of this afferent to repeated BK by 71% (1.73 to 2.95 impulses/s). Baseline activity of this afferent nerve was not altered by naloxone.

The responses of two groups of cardiac afferent nerves to 1 \(\mu\)g BK are shown in Fig. 6, B and C, and Table 1. In the first group, the initial application of BK significantly increased the discharge activity of seven unmyelinated afferents (\(CV = 0.60 \pm 0.08\) m/s) from 0.92 ± 0.10 to 1.97 ± 0.19 impulses/s (Fig. 6B). The BK-induced increases in the activity of these afferent nerves were facilitated by 85% after blockade of opioid receptors with epicardial 8 \(\mu\)mol naloxone (1.26 ± 0.21 vs. 2.3 ± 0.42 impulses/s, initial vs. repeated BK, \(P < 0.05\)). Naloxone also enhanced the response duration as well as total responses of afferent nerves to BK (Table 1). The MAP response was unchanged during the initial and repeat applications of BK (initial: 96 ± 9 vs. 107 ± 7 mmHg and repeat: 98 ± 8 vs. 105 ± 7 mmHg, before vs. after BK, both \(P > 0.05\)). Naloxone did not change afferent nerve baseline activity. In the second group, seven other cardiac afferents (one A\(\delta\)-fiber, \(CV = 3.29\) m/s, and six C-fibers, \(CV = 0.57 \pm 0.12\) m/s) responded consistently to BK after the administration of vehicle (PBS; Fig. 5B). The locations of the 14 afferent nerve endings in this protocol are shown in Fig. 1.

**DISCUSSION**

Two novel observations were made in the present study. First, we found that nonselective blockade of opioid receptors with epicardial naloxone enhanced the responses of cardiac spinal afferent nerves to myocardial ischemia. Second, administration of naloxone significantly facilitated the response of ischemically sensitive cardiac afferent nerves to stimulation with epicardial ATP and BK. Naloxone itself did not alter baseline activity of cardiac spinal afferent nerves. Thus, these data strongly suggest that endogenously produced opioid peptides inhibit cardiac spinal afferent nerve activity during myocardial ischemia by modulating the responses of these afferent nerves to ischemic mediators like ATP and BK.

Three families of endogenous opioid peptides are well characterized within the central and peripheral nervous systems. The principal representatives of each family are \(\beta\)-endorphin, met- and leu-enkephalin, and dynorphin. The inhibitory role of these opioids in the central nervous system is well known (34, 35, 62, 63, 69). However, the functional influence of opioids on
the peripheral nervous system is unclear. Recent studies have provided several lines of evidence suggesting that opioid peptides play crucial roles in the regulation of cardiac sympathetic afferent nerve activity during myocardial ischemia. First, opioids and their precursors (proproenkephalin, prodynorphin, and propiomelanocortin) are synthesized, stored, and released by cardiac myocytes, sympathetic nerves, and both polymorphonuclear and mononuclear leukocytes, including monocytes/macrophages and lymphocytes (38, 41, 44, 67). Leukocytes accumulate in the coronary arterial plaques and release large concentrations of opioids when they are activated during plaque disruption, resulting in myocardial ischemia and infarction (12, 23, 36, 46). For instance, leukocytes accumulate continuously during atheroma formation, especially in intimal atherosclerotic lesions (23, 60). Activated leukocytes are abundant at the sites of coronary plaque rupture (36, 65). Plasma enkephalin and \( \beta \)-endorphin are increased during acute myocardial ischemia in rats (40). Several clinical studies (5, 7, 27) have demonstrated that, compared with \( \beta \)-endorphin in the femoral arterial blood, coronary sinus concentrations are dramatically higher (15 ± 4 vs. 75 ± 17, femoral vs. coronary) in patients experiencing myocardial ischemia and/or infarction who are treated with transluminal angioplasty. Second, anatomic studies (13, 24, 29, 42, 59) have demonstrated that \( \mu \)-, \( \delta \)-, and \( \kappa \)-opioid receptors are located on small-, medium-, and large-diameter sensory neurons in the DRG, nodose, and trigeminal ganglia of animals and humans. Receptor proteins are transported to nerve terminals after being synthesized in their perikarya (29, 38, 44, 67). Hence, the results of these studies suggest that endogenous opioid peptides released during myocardial ischemia are potentially available to regulate the excitability of cardiac sympathetic afferent nerve during ischemia through the activation of opioid receptors located in sensory nerve endings.

Opioid \( \mu \)-, \( \delta \)-, and \( \kappa \)-receptors in the peripheral nervous system may play an important physiological role, although virtually all previous studies have involved the pharmacological application of opioid agonists (59). Furthermore, variable somatic and vagal sensory neural responses to the activation of opioid receptors have been reported (2, 9, 10, 25, 30, 52). In this regard, the \( \mu \)-receptor agonist [\( D-Ala^2, N-MePhe^4 \), Gly-ol]-enkephalin (DAMGO) directly excites rat small intestinal afferent nerves (11). Similarly, activation of peripheral opioid \( \mu \)-, \( \delta \)-, and \( \kappa \)- receptors increases the discharge activity of testis-spermatic sensory nerves of dogs (30). Moreover, opioid receptor ligands excite rat mesenteric afferent nerve fibers and mouse DRG neurons, responses that can be eliminated by opioid receptor antagonism (25, 54). In contrast, other studies (48, 54, 58) have shown that activation of opioid receptors on pelvic and gastric vagal afferent nerves modulates visceral

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**Fig. 6.** A: neurohistograms showing responses of left ventricular afferent nerves (posterior wall, CV = 0.55 m/s) to epicardial application of BK before (1) and after (2) treatment with naloxone. B: responses of seven cardiac sympathetic afferents to epicardial application of BK before and after treatment with naloxone. C: consistent responses of seven other cardiac afferent nerves to BK. Values are means ± SE. *P < 0.05 compared with control; #P < 0.05 postnaloxone vs. prenaloxone.
pain. Approximately 58% of cutaneous C- and Aδ-sensory nerve fibers are opiate sensitive, and the application of μ-agonists like morphine inhibits their discharge activity (68). Stimulation of peripheral μ- and δ-opioid receptors also inhibits chemically induced visceral and somatic nociception (2, 9, 10, 52), whereas opioid receptor blockade with naloxone eliminates the action of opioids on these polymodal nociceptors (2, 48, 53). Because these studies are pharmacological, it has been uncertain if endogenous opioids play a role in afferent nerve activation or inhibition in physiological or pathophysiological conditions when they are released and have the potential to exert independent actions or effects in concert with other mediators. Thus, the present study provides the first evidence demonstrating that endogenously produced opioids modulate cardiac sensory nerve discharge, in this case the heightened activity of cardiac sympathetic afferent nerves associated with myocardial ischemia.

A number of mediators released during myocardial ischemia excite cardiac spinal afferent nerves (19, 37). BK, for example, stimulates cardiac afferent nerves with cell bodies in the DRG through interactions with BK2 receptors (61, 70). BK2 receptors belong to a family of seven transmembrane GPCRs. Neuronal BK2 GPCRs, coupled to Gq protein, excite a phospholipase C-PKC pathway, leading to the activation of phospholipase A2 and ultimately prostaglandin generation, which, in turn, activates a cAMP-PKA pathway to generate action potentials (32, 61, 70). Opioid μ-, δ-, and κ-receptors likewise belong to the large family of GPCRs. However, they specifically use Gi and/or Gq subfamily members to inhibit adenyl cyclase generation of cAMP (66). Because these studies are pharmacological, it has been uncertain if endogenous opioids play a role in afferent nerve discharge and/or neurotransmitter release in many neuronal systems; and

Additionally, BK2 receptors colocalize with opioid receptors in trigeminal ganglion neurons (4). Hence, there is a strong potential for endogenous opioids to modulate (rather than excite) responses of cardiac spinal afferent nerves to BK during ischemia through shared GPCRs coupled to various cellular signaling pathways, particularly opioid GPCRs coupled to Gi/Go, which counteract the actions of BK2 GPCRs coupled to Gq. In support of our observations, a pharmacological study (51) has reported that the μ- and δ-receptor agonists DAMGO and [d-Ala², d-Leu²]-enkephalin (DADLE) suppress PGE₂-stimulated cAMP accumulation by 50–75% in trigeminal ganglion neurons (4, 51) and modulate PGE₂-induced hyperalgesia in primary afferent nociceptors by inhibiting an intracellular cAMP-PKA-vanilloid receptor 1 pathway in rats (33, 66). Additionally, BK2 receptors colocalize with opioid receptors in trigeminal ganglion neurons (4). Hence, there is a strong potential for endogenous opioids to modulate (rather than excite) responses of cardiac spinal afferent nerves to BK during ischemia through shared GPCRs coupled to various cellular signaling pathways, particularly opioid GPCRs coupled to Gi/Go, which counteract the actions of BK2 GPCRs coupled to Gq. In support of our observations, a pharmacological study (51) has reported that the μ- and δ-receptor agonists DAMGO and DADLE inhibit BK-evoked cGRP release in trigeminal ganglia through a BK2 GPCR mechanism.

Extracellular ATP is another ischemic mediator that specifically stimulates ischemically sensitive cardiac spinal afferent nerves but not ischemically insensitive endings, through a purinergic (P2) receptor mechanism that involves both P2X and P2Y receptors (20). P2X receptors activate ligand-gated ion channels, whereas P2Y receptors exert their actions through a GPCR mechanism (6). In the present study, we confirmed our hypothesis that endogenous opioid peptides modulate the responses of cardiac spinal afferent nerves to extracellular ATP. In agreement with our data, other investigators (8) have observed that the μ-receptor agonist leu-enkephalin and morphine inhibit ATP-evoked excitation of somatic C-fiber afferent nerves in rats through a G protein-dependent mechanism, an effect that can be reversed by naloxone. Excitation of opioid receptors also suppresses ATP-activated currents in nodose and DRG neurons. This response can be reversed by the addition of GDP to the intracellular solution (8, 71), which prevents binding of GTP to the activated G protein and subsequently blocks signal transduction from the Gq/Go protein-coupled opioid receptor complex (56).

In summary, the present study provides novel evidence demonstrating that endogenously produced opioids suppress the responses of cardiac spinal afferent nerves to ischemia and to the excitatory ischemic mediators BK and ATP. The interactions between endogenous opioids and excitatory mediators like BK and ATP contribute to the net response of cardiac afferent nerves during myocardial ischemia. These findings suggest that endogenously produced opioids may protect the heart during ischemia by reducing the magnitude of excitatory cardiovascular reflex responses (22) that increase the imbalance between myocardial O₂ supply and demand and hence exacerbate ischemia.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: L.-W.F. conception and design of research; L.-W.F. performed experiments; L.-W.F. analyzed data; L.-W.F. and J.C.L. interpreted results of experiments; L.-W.F. prepared figures; L.-W.F. drafted manuscript; L.-W.F. and J.C.L. edited and revised manuscript; L.-W.F. and J.C.L. approved final version of manuscript.

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