Endogenous tachykinins cause bradycardia by stimulating cholinergic neurons in the isolated guinea pig heart

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MATERIALS AND METHODS

Isolated heart preparation. Male Hartley guinea pigs (350–450 g) were pretreated with 500 U of heparin before undergoing decapitation while anesthetized with pentobarbital sodium (75 mg/kg ip). The heart was rapidly removed and placed in ice-cold perfusion buffer to enable cannulation of the ascending aorta. After flushing the heart with 5 ml of cold

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buffer, it was transferred to an isolated heart apparatus for perfusion by a modification of the Langendorff technique (4). The perfusion solution was a modified Krebs-Ringer bicarbonate buffer (pH 7.35–7.4) of the following composition (in mM): 127.2 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 K,H₂PO₄, 1.2 MgSO₄, 2.0 sodium pyruvate, 5.5 dextrose, and 0.1% BSA. A Masterflex peristaltic pump (Cole Parmer, St. Louis, MO) was used to perfuse spontaneously beating hearts at a constant rate of 8 ml/min. Buffer in the reservoir was continuously gassed with 95% O₂:5% CO₂. The temperature of the buffer was maintained at 37°C. Cardiac contractions were measured by attaching one end of a silk suture to the apex of the heart and the other end to an isometric force transducer. Diastolic tension on the heart was adjusted to −1 g. Output from the force transducer was sent to a Gould Universal amplifier and a Gould Biotach amplifier to monitor ventricular contractions and heart rate, respectively, with a Gould 2400 recorder. Because perfusion rate was held constant, perfusion pressure was monitored as an indicator of coronary vascular resistance. This parameter was recorded using a pressure transducer that was attached to the sidearm of a three-way stopcock located at the proximal end of the aortic cannula. Experiments were started after a 40-min stabilization period.

Pacing. To evaluate inotropic responses in the absence of rate changes, hearts were paced in some experiments using an SD9 stimulator (Grass Instruments, Quincy, MA). Pacing wires with platinum needle electrodes were attached to the right atrium and apex of the heart. Stimulation parameters were 2 V, 10-ms duration, and 2-ms delay at a frequency to clamp heart rate at ~50 beats/min above the spontaneous rate. The perfusion rate was increased to 12 ml/min during pacing to keep the amplitude of ventricular contraction at a level near that observed during spontaneous beating.

Preparation and storage of drugs. NKA, human α-CGRP, ACh, CGRP-(8—37), neostigmine methyl sulfate, and atropine were dissolved in saline. Capsaicin was dissolved in saline with 10% ethanol. Aliquots of CGRP, CGRP-(8—37), and capsaicin were stored at 80°C. Neostigmine methyl sulfate, atropine, and ACh were weighed and diluted before each experiment. CGRP and CGRP-(8—37) were diluted with saline that contained 0.1% BSA. Capsaicin was diluted with saline.

Drug administration. Bolus injections and infusions were made into PE-20 tubing that emptied into the perfusion line at a point immediately above the three-way stopcock used for attachment to the pressure transducer. Capsaicin (100 nmol), CGRP (0.32 nmol), and ACh (1 nmol) were administered by bolus injection in a volume of 100 µl over about 3 s. The CGRP antagonist CGRP-(8—37) was infused at 100 µl/min to achieve a final concentration of 1 µM at the heart. Infusions of CGRP-(8—37) were started 5 min before injection of capsaicin or CGRP and continued for 10 min. NKA was given by infusion at 25 nmol/50 µl/h to desensitize tachykinin receptors (18). This achieved a final concentration of 3.12 µM NKA at the heart. Capsaicin was injected 10 min after starting the infusion of NKA when desensitization to tachykinins was well established. Neostigmine (0.5 µM) was used to inhibit cholinesterases, and muscarinic receptor blockade was established with atropine (1 µM). Neostigmine and atropine were administered by including these drugs in the perfusion buffer. The perfusion was switched to buffer containing neostigmine alone or neostigmine in combination with atropine at least 20 min before challenge with capsaicin.

Experimental protocol for evaluating responses to capsaicin. Tachyphylaxis can occur in response to capsaicin, so only one injection of this drug was administered to each heart. Responses to a single bolus injection of capsaicin were determined in five groups of spontaneously beating hearts. One group was used to determine normal responses to capsaicin (i.e., control). The effect of specific treatments on the responses to capsaicin were evaluated in the remaining groups as follows: 1) CGRP-(8—37), 2) CGRP-(8—37) + neostigmine, 3) CGRP-(8—37) + neostigmine + NKA infusion, and 4) CGRP-(8—37) + neostigmine + atropine.

Experimental protocols for evaluating responses to CGRP. After recording responses to capsaicin, five of the control hearts were also used to quantify responses to a bolus injection of 0.32 nmol CGRP and determine the effect of CGRP-(8—37) on responses to CGRP. An additional six hearts were used to determine if the negative inotropic effect of CGRP in spontaneously beating hearts also occurred when hearts were paced.

Data analysis. Heart rate (beats/min), diastolic perfusion pressure (mmHg), and force of ventricular contractions (g) were measured at baseline and at times of maximum responses to capsaicin, CGRP, and ACh. Baseline and response values were compared using a paired t-test. Percent changes in values relative to baseline were calculated, and between-group comparison of these values was done by one-way ANOVA. Graphing of data and statistical analyses were performed using GraphPad Prism version 3.01 (GraphPad Software, San Diego, CA). Group data are summarized as arithmetic means ± SE. The Newman-Keuls procedure was used for post hoc comparisons when indicated after one-way ANOVA. A probability level of 0.05 or smaller was used to indicate statistical significance.

Drugs. NKA was purchased from Peninsula (Belmont, CA). Capsaicin, neostigmine methyl sulfate, atropine sulfate, and ACh chloride were purchased from Sigma (St. Louis, MO). Human α-CGRP and CGRP-(8—37) were purchased from Bachem Bioscience (King of Prussia, PA).

RESULTS

Responses to capsaicin and effects of CGRP-(8—37). Administration of 100 nmol of capsaicin caused very prominent cardiac responses in untreated, control hearts (see Figs. 1A and 3). Heart rate increased from a baseline of 257 ± 14 to a maximum of 319 ± 10 beats/min (n = 6, P < 0.01). This response was prolonged, requiring 420 ± 78 s for 80% recovery. The tachycardia was accompanied by a decrease in ventricular contractility that followed a similar time course. The negative inotropic response comprised a decrease in the peak force of ventricular contractions and, in five of six hearts, an increase in the tension at the apex of the heart during diastole (Fig. 1A). Capsaicin also caused a small decrease in diastolic perfusion pressure from a baseline of 26 ± 1 to a minimum of 24 ± 1 mmHg (P < 0.05, Fig. 1A).

Infusion of CGRP-(8—37) at a dose producing a final concentration of 1 µM at the heart had no effect on baseline parameters but reduced cardiac responses to 100 nmol capsaicin (see Figs. 1 and 3). The positive chronotropic response to capsaicin was eliminated in the presence of the CGRP receptor antagonist and replaced by a minor decrease in heart rate from a baseline of 252 ± 9 to a minimum of 246 ± 9 beats/min (n = 6, P < 0.05). Although capsaicin still decreased ventricular contractility in the presence of CGRP-(8—37), the percent decrease was significantly reduced.
compared with the value obtained in untreated hearts (−23.5 ± 4.6% for control compared with −7.2 ± 2.0% with CGRP-(8—37) present; P < 0.01, unpaired t-test, Fig. 1). Furthermore, the effect of capsaicin to increase diastolic tension at the apex of the heart was eliminated by the CGRP antagonist (Fig. 1). The small coronary vasodepressor response to capsaicin was unaffected by CGRP-(8—37) (P = 0.82, unpaired t-test).

Responses to CGRP. Bolus injections of 0.32 nmol CGRP produced a response pattern very similar to that evoked by capsaicin, increasing heart rate and decreasing contractility and perfusion pressure (Table 1). The decrease in contractility evoked by CGRP comprised a decrease in maximum tension and an increase in diastolic tension at the apex, as observed with capsaicin. The CGRP antagonist caused a marked attenuation of all responses to CGRP (Table 1).

Responses to CGRP and ACh were evaluated in another group of hearts while they were beating spontaneously and again while they were paced (Table 2). CGRP and ACh had opposite effects on heart rate, but both agents reduced ventricular contractility and perfusion pressure. Negative inotropic and coronary vasodilator responses to CGRP and ACh were unaffected by pacing.

Effect of neostigmine on responses to capsaicin in the presence of CGRP-(8—37). Baseline heart rate was reduced from 252 ± 7 to 214 ± 6 beats/min during exposure to 0.5 µM neostigmine. Ventricular contractility was also decreased slightly in six of eight hearts, but this change was not statistically significant (2-tailed, paired t-test).

Table 1. Effect of the CGRP receptor antagonist CGRP-(8—37) on responses of isolated perfused guinea pig hearts to a bolus injection of 0.32 nmol CGRP

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CGRP</th>
<th>CGRP + 1 µM CGRP-(8—37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate</td>
<td>23.9 ± 2.4</td>
<td>4.9 ± 1.3*</td>
</tr>
<tr>
<td>Ventricular contractility</td>
<td>−23.1 ± 3.2</td>
<td>−6.2 ± 2.7†</td>
</tr>
<tr>
<td>Perfusion pressure</td>
<td>−10.8 ± 1.6</td>
<td>−2.6 ± 1.1†</td>
</tr>
</tbody>
</table>

Values are means ± SE change in recorded parameter (% baseline) (n = 5). Paired t-test was used to compare changes produced by calcitonin gene-related peptide (CGRP) alone and in presence of CGRP antagonist. *P < 0.01, †P < 0.001, ‡P < 0.05.

Table 2. Effect of pacing on responses of isolated guinea pig hearts to bolus injections of CGRP and ACh

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Spontaneous Beating</th>
<th>Paced</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGRP (0.32 nmol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate</td>
<td>25.0 ± 3.2</td>
<td>22.5 ± 5.5</td>
</tr>
<tr>
<td>Ventricular contractility</td>
<td>−23.1 ± 6.4</td>
<td>−22.8 ± 6.8</td>
</tr>
<tr>
<td>Perfusion pressure</td>
<td>−12.6 ± 3.8</td>
<td>−15.9 ± 3.8</td>
</tr>
</tbody>
</table>

| ACh (1 nmol)               |                     |             |
| Heart rate                 | −77.0 ± 5.2         | −75.5 ± 6.2 |
| Ventricular contractility  | −18.0 ± 1.6         | −20.3 ± 2.7 |
| Perfusion pressure         | −29.7 ± 2.9         | −22.3 ± 4.4 |

Values are means ± SE change in recorded parameter (% baseline) (n = 5 or 6). No significant differences were detected by using a 2-tailed, paired t-test to compare responses recorded while hearts were beating spontaneously and paced.
paired t-test; \( P = 0.15 \)). Perfusion pressure was unaffected by the cholinesterase inhibitor. While hearts were subjected to continued exposure to neostigmine and infusion of the CGRP antagonist, bolus injections of 100 nmol capsaicin produced a large and prolonged bradycardia (Figs. 2 and 3). Heart rate decreased from a baseline of 214 ± 6 to a minimum of 153 ± 22 beats/min \( (P < 0.05, \) paired t-test, \( n = 8 \)). The interval from the initial decrease in heart rate until 80% recovery was 185 ± 24 s. Although small increases in ventricular contractility were evident in some recordings (Fig. 2), the average maximum did not differ from baseline \( (7.31 ± 0.99 \text{ compared with } 7.65 ± 0.68 \text{ g after capsaicin}; \ P = 0.49, \) paired t-test, \( n = 8 \)). Likewise, small decreases in perfusion pressure did not achieve statistical significance when baseline and minimum perfusion pressure after capsaicin were compared \( (25 ± 3 \text{ vs. } 20 ± 1 \text{ mmHg}; \ P = 0.052, \) paired t-test, \( n = 8 \)).

Effects of NKA infusion and atropine on responses to capsaicin. Each of these treatments was done in separate hearts with the CGRP antagonist and neostigmine present. NKA was infused in one group of hearts to desensitize tachykinin receptors. Under this condition, capsaicin produced a small decrease in heart rate relative to baseline in four of six hearts (Fig. 2B). However, this change was not statistically significant for the group \( (205 ± 4 \text{ at baseline vs. } 185 ± 11 \text{ beats/min after capsaicin, } \ P = 0.12, \) paired t-test, \( n = 6 \)). Capsaicin given while tachykinin receptors were desensitized had no significant effect on ventricular contractility \( (7.00 ± 0.69 \text{ vs. } 7.55 ± 0.70 \text{ g after capsaicin}; \ P = 0.28) \) or perfusion pressure \( (23.3 ± 1.1 \text{ vs. } 23.0 ± 1.1 \text{ mmHg after capsaicin}; \ P = 0.17) \). Atropine did not affect baseline cardiac parameters or perfusion pressure. In the presence of atropine, capsaicin had no effect on heart rate, ventricular contractility, or perfusion pressure.

DISCUSSION

Results from this study provide the first evidence that endogenous tachykinins can produce bradycardia in the guinea pig through stimulation of cholinergic neurons. Previous experiments with isolated guinea pig hearts (17) and anesthetized guinea pigs (33) demonstrated that intracardiac cholinergic neurons could be stimulated by the administration of exogenous SP. Furthermore, microelectrode studies of isolated guinea pig intracardiac ganglia established that exogenously applied tachykinins produce a slow depolarization of cardiac neurons and increase their excitability \( (15, 16, 23) \). There is also evidence that endogenous tachykinins, released by application of capsaicin or stimulation of intraganglionic nerves, have a similar effect \( (15, 16, 23) \). However, selective stimulation of tachykinin-containing afferent fibers innervating the intrinsic cardiac ganglia is not feasible at present, and chemical stimulation of cardiac afferents in isolated atria or perfused hearts evokes cardiac responses dominated by CGRP \( (8) \). Blockade of CGRP receptors with CGRP-(8—37) and potentiation of cholinergic responses with neostigmine were essential for unmasking...
provides strong evidence implicating endogenous tachykinins in the negative chronotropic response. Complete blockade of the response by atropine implicates cholinergic neurons. Although the effects of atropine and desensitization were not significantly different, it should be noted that a small bradycardic response remained in some hearts with tachykinin receptors desensitized. It is possible that this residual bradycardia may reflect incomplete tachykinin receptor desensitization in these preparations. Alternately, an additional mediator that activates cholinergic neurons might be released from some cardiac afferents by capsaicin.

Cholecystokinin and dynorphin are colocalized with the tachykinins and CGRP in some neurons of the dorsal root ganglia in guinea pigs, but these peptides are not present in capsaicin-sensitive neurons that innervate the heart (14). In contrast, pituitary adenylate cyclase-activating peptide (PACAP) is present in nerve fibers that innervate intrinsic cardiac ganglia of the guinea pig (3) and rat (24). This peptide is also expressed by some capsaicin-sensitive neurons in rat dorsal root ganglia (29). Whether PACAP is colocalized with the tachykinins and CGRP in cardiac afferents of the guinea pig is presently unknown. However, it is known that exogenously applied PACAP increases the excitability of guinea pig intracardiac neurons in vitro (3). If PACAP is released by capsaicin, the present findings suggest that its influence would be small compared with that of the tachykinins.

Although vagal stimulation can reduce ventricular contractility as well as heart rate by activating cholinergic neurons in the intrinsic cardiac ganglia (1), endogenous tachykinins only evoked bradycardia in the present study. Because the magnitude of negative inotropic response to vagal stimulation is enhanced during sympathetic nerve stimulation (25), it is possible that the absence of sympathetic tone in the isolated guinea pig heart preparation prevented the expression of a negative inotropic response mediated by cholinergic neurons. In this regard, it should be noted that bolus injection of ACh caused a larger decrease in heart rate than ventricular contractility. It is also possible that the prominent bradycardia evoked by capsaicin, in the presence of neostigmine and CGRP receptor blockade, secondarily affected ventricular contractility to mask a negative inotropic response. Previous work has demonstrated that such an effect occurs with NKA in the isolated guinea pig heart (18). Because intrinsic cardiac ganglia exhibit some degree of specialization in regulating different aspects of cardiac functions (1), it is also possible but less likely that endogenous tachykinins do not affect cholinergic neurons that control ventricular contractility.

Previous investigators have reported that CGRP-(8–37) inhibits positive inotropic responses to CGRP, capsaicin, and electrical field stimulation in isolated guinea pig atria (7, 27), as well as the positive chronotropic response to capsaicin in anesthetized guinea pigs (32), but the effect of this blocker on responses of perfused guinea pig hearts has not been reported. In the present study, we took advantage of evidence that CGRP-(8–37) associates with the CGRP receptor very
rapidly (34). This allowed us to block CGRP receptors by starting infusion of CGRP-(8–37) only 5 min before injection of capsaicin. In addition to blocking positive chronotropic responses to capsaicin and CGRP, the CGRP antagonist eliminated the negative inotropic response to CGRP and attenuated this effect from capsaicin. Other investigators have reported that capsaicin produces a negative inotropic effect independent of its action on cardiac afferents (8, 11, 37). Our results are in part consistent with this conclusion, because a portion of the negative inotropic response to capsaicin remained in the presence of CGRP-(8–37). However, our data also indicate that exogenous and endogenous CGRP can decrease ventricular contractility. The simplest explanation for this response would be that it occurs secondarily to excessive oxygen demand caused by drug-evoked tachycardia. This would be consistent with reports that CGRP does not directly affect guinea pig ventricular myocardium (20). However, our observation that the negative inotropic response to CGRP persists during pacing suggests that CGRP might affect ventricular function by a more direct mechanism. This possibility is also supported by our recent autoradiographic identification of specific CGRP receptors in ventricular myocardium of the guinea pig (5).

CGRP is known to have a potent coronary vasodilator action in several species (2), and this effect can be inhibited by CGRP-(8–37) (9). Although capsaicin decreased perfusion pressure in the present study, this response was not influenced by CGRP-(8–37), whereas vasodilatation to CGRP was attenuated. These observations suggest that CGRP does not contribute to the small vasodilator effect of capsaicin in our preparations. However, coronary vascular tone is low under our experimental conditions, and the sensitivity for detecting coronary vasodilatation is less than optimum. Accordingly, additional studies will be needed to elucidate effects of endogenous sensory neuropeptides on the coronary vasculature of guinea pig.

Perspectives

Release of the tachykinins and CGRP from cardiac afferents occurs during ischemia in response to low pH and mediators such as bradykinin and prostaglandins (10–12). These stimuli also initiate action potentials that travel to collateral processes and to central nerve endings of the same neurons (8, 21, 28, 30). Neuropeptides released from afferent endings in the central nervous system are believed to mediate cardiac pain, whereas those released from collaterals mediate axon reflexes. Although no information is available regarding the arborization of nerve processes from single sensory neurons within the heart, it is reasonable to speculate that the tachykinin-containing nerve fibers surrounding neurons of the intrinsic cardiac ganglia are collaterals of sensory neurons with primary projections to the myocardium or coronary vasculature. Accordingly, activation of these intracardiac and perivascular nerve endings during myocardial ischemia or infarction would trigger an axon reflex that releases tachykinins from collaterals in the intrinsic cardiac ganglia. Electrophysiological evidence has established that tachykinins can increase the excitability of intracardiac neurons (15, 16, 23), thereby lowering their threshold for activation by preganglionic vagal input. Tachykinins can also evoke spontaneous firing of intracardiac neurons (15, 16). The present results support the hypothesis of an intracardiac axon reflex by demonstrating that endogenous tachykinins can affect cardiac function through stimulation of cholinergic neurons in the heart. This reflex would function to decrease heart rate and cardiac work during ischemia.

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REFERENCES


