Calcitonin Gene-Related Peptide in Noradrenergic Transmission in Rat Hypothalamus

Kazushi Tsuda, Seiko Tsuda, Menek Goldstein, Ichiro Nishio, and Yoshiaki Masuyama

In the present study, we examined the regulatory mechanisms of calcitonin gene-related peptide on norepinephrine release in rat hypothalamus. Calcitonin gene-related peptide inhibited the stimulation-evoked norepinephrine release from hypothalamic slices of Sprague-Dawley rats in a dose-dependent manner, although the peptide did not affect basal release of norepinephrine. The blockade of the α2-adrenergic receptors by RX 781094 failed to modulate the inhibitory effects of calcitonin gene-related peptide on norepinephrine release. Pretreatment of slices with islet activating protein, a toxin that interferes with the coupling of the inhibitory receptors to adenylate cyclase, did not affect the suppression of norepinephrine release by calcitonin gene-related peptide. However, Bay K 8644, a dihydropyridine-sensitive calcium channel agonist, significantly reversed the inhibitory effects of calcitonin gene-related peptide on norepinephrine release. These results show that calcitonin gene-related peptide might inhibit norepinephrine release in rat hypothalamus, partially mediated by interactions with dihydropyridine-sensitive Ca2+ channels but not by interactions with presynaptic α2-adrenergic receptors and inhibitory guanosine triphosphate binding proteins. Furthermore, the finding suggests the possible involvement of calcitonin gene-related peptide in the regulation of sympathetic nervous activity in the central nervous system. (Hypertension 1992;19:639–642)

KEY WORDS • calcitonin gene-related peptide • norepinephrine • hypothalamus • adrenergic receptors • pertussis toxins • Bay K 8644

calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide that is encoded by the same gene as the precursor of calcitonin and is produced by alternative processing of the messenger RNA (mRNA) transcribed from the calcitonin gene.1,2 In immunohistochemical studies, it has been shown that CGRP is widely distributed in both the central and peripheral nervous systems.2,3 The presence of a high level of CGRP immunoreactivity has been demonstrated in the central amygdaloid, caudate putamen, spinal trigeminal nucleus and tract, substantia gelatinosa, dorsal horn of spinal cord, hypothalamus, and medulla oblongata containing nucleus tractus solitarii in rats.3 These findings suggest that CGRP might have a crucial role in the regulation of sensory, motor, and autonomic nerve functions. It has been reported that intracerebroventricular administration of CGRP produced a dose-dependent increase in blood pressure and heart rate in rats, which suggested that CGRP might act to modulate the noradrenergic sympathetic outflow in the brain.4 In the peripheral tissues, Ohhashi and Jacobowitz5 observed that CGRP inhibited the electrical stimulation-induced contraction of rat vas deferens, which indicated that CGRP might inhibit the release of norepinephrine during adrenergic nerve stimulation. However, functional significance and mechanisms of the CGRP effects in noradrenergic transmission remain to be elucidated. In the present study, we studied the regulatory mechanisms of CGRP on norepinephrine release in rat hypothalamus and determined a possible role of CGRP in the regulation of noradrenergic transmission in the central nervous system.

Methods

Male Sprague-Dawley rats (200–250 g, Taconic Farms, Inc., Germantown, N.Y.) were used in the experiment. Rats were fed regular pellet food and tap water ad libitum beginning at least 1 week before the experiment. Rats were killed by decapitation, and the whole hypothalamus was rapidly dissected on ice according to the method described previously.6 The isolated hypothalamus was sliced at 0.3-mm thickness by means of a tissue chopper (Brinkmann Instruments, Inc., Westbury, N.Y.), rotated 90°, and sliced again (0.3×0.3 mm). The sliced tissues were washed three times with 2 ml of Krebs-Ringer bicarbonate buffer (mmol/l: NaCl 118.0, KCl 4.80, CaCl2 1.20, KH2PO4 1.15, MgSO4 1.20, NaHCO3 25.9, glucose 11.1, ascorbic
were incubated with 3 ml of fresh buffer containing 0.1 μM of [3H]norepinephrine (specific activity, 40.8 Ci/ mmol, New England Nuclear Research Products, Boston, Mass.) for 20 minutes at 37°C. After the slices (5–7 mg) were rinsed with fresh buffer, they were transferred to a superfusion chamber (200 μl), jacketed with 37°C water, and suspended between two platinum electrodes (25 mm apart, 2.0 mm long). Slices were superfused at a rate of 0.7 ml/min with Krebs-Ringer bicarbonate buffer. Sample collection began after 60 minutes of superfusion, when basal outflow of tritium had stabilized to a constant level. Samples of superfusate were collected at 7-minute intervals until the end of the experiment (130 minutes). The first period of electrical stimulation (S1) was applied at 67 minutes, and the second period of electrical stimulation (S2) was applied at 116 minutes after the beginning of the superfusion.

Electrical stimulation was delivered by a stimulator (model S4K, Grass Instrument Co., Quincy, Mass.) and consisted of trains of unipolar, rectangular pulses (1 Hz, 20 mA, 2-msec duration) for 2 minutes. At the end of the experiment, the slices were sonicated for 20 seconds. Radioactivity in collected samples and tissue slices was determined by liquid scintillation counting (Tricarb liquid scintillation spectrometer 3255, Packard Instrument Co., Inc., Sterling, Va.). The amount of radioactivity in each sample was calculated by dividing the total tritium collected in each sample by the total tritium present in the tissue at the time of sample collection (percent fractional release). Basal outflow during the two prestimulation periods (b1 and b2, respectively) was calculated from the tritium collected in the two collected samples just before S1 and S2. The stimulation-evoked release was calculated by subtracting the basal outflow during the 7-minute prestimulation period from the value in the stimulation period, which included 2 minutes during stimulation plus 5 minutes after stimulation (total, 7 minutes).

We first examined the effects of CGRP alone on norepinephrine release in rat hypothalamic slices. In the control experiment, S1 and S2 were performed in the absence of the peptide. The effects of CGRP were evaluated only in S2, with S1 as an internal control. Superfusion of CGRP was initiated 14 minutes before S2 and maintained until the end of the experiment. The effects of CGRP on stimulation-evoked and basal release of [3H]norepinephrine were determined by comparing the S2/S1 and b2/b1 ratios obtained in control slices with the values in slices treated with CGRP. To examine the effects of the blockade of α2-adrenergic receptors, we added RX 781094 (1 x 10^-8 M) to the superfusion medium 28 minutes before S1 and maintained it until the end of the experiment. Then, to inactivate the inhibitory guanosine triphosphate binding protein (G protein), we incubated the slices for 1 hour at 37°C in an atmosphere of 95% O2-5% CO2 in a mixture of 920 μl of Krebs-Ringer bicarbonate buffer and 80 μl of 0.01 M sodium phosphate buffer that contained 0.05 M NaCl and 8 μg of pertussis toxin (islet activating protein [IAP]). For the control experiment, slices were incubated in the same buffer mixture without IAP. Subsequently, the slices were washed three times with fresh buffer and incubated in the presence of 0.1 μM [3H]norepinephrine for 20 minutes at 37°C, as previously described. In the third series of experiments, we investigated the effects of CGRP on hypothalamic norepinephrine release in combination with Bay K 8644, a dihydropyridine-sensitive Ca2+ channel agonist. Bay K 8644 and CGRP were added to the superfusion medium 14 minutes before S2 and maintained until the end of the experiment. Bay K 8644 was dissolved in ethanol (95% vol/vol) and diluted in Krebs-Ringer bicarbonate buffer just before the experiment. For the control experiment, the same volume of ethanol was added in the medium.

**Drugs**

CGRP (α-type) was purchased from the Protein Institute Inc., Osaka, Japan. The α2-antagonist 2-(1,4-benzodiozanyl)-2-imidazolin-HCl (RX 781094) was received from RPI Corp., Mt. Prospect, Ill. Purified IAP was obtained from List Biological Laboratories, Inc., Campbell, Calif. Bay K 8644, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl-phenyl)-pyridine-5-carboxylate, was kindly donated by Miles Pharmaceuticals, West Haven, Conn., and assiduously protected from light throughout the experiment. All other chemicals and reagents were from commercial sources and were of the highest purity available.

**Statistics**

Data are presented as mean±SEM. Differences between the means of the drug treatment and their corresponding controls were tested with one-way analysis of variance. To compare the means of the different study groups, we performed statistical analyses with the Mann-Whitney U test. A value of p<0.05 was accepted as the level of significance.

**Results**

**Effects of Calcitonin Gene-Related Peptide Alone and in Combination With RX 781094 on the Stimulation-Evoked Release of Tritiated Norepinephrine**

Table 1 shows the effects of CGRP on electrical stimulation-evoked [3H]norepinephrine release in rat hypothalamic slices. CGRP inhibited the stimulation-evoked [3H]norepinephrine release in a concentration-dependent manner (IC50 value, 5.1±1.7 x 10^-8 M, n=8). The basal release of tritium (b2/b1 ratio) was slightly increased by the high concentrations of the peptide, but the changes were not significant (Table 1). Exposure of the hypothalamic slices to RX 781094 before the period of S1 increased the stimulation-evoked release of [3H]norepinephrine. Under this experimental condition, CGRP inhibited stimulation-evoked norepinephrine release by a magnitude similar to that produced by CGRP alone in the absence of RX 781094 (Table 1).

**Effects of Pretreatment of Slices With Islet Activating Protein on the Inhibition of Tritiated Norepinephrine Release by Calcitonin Gene-Related Peptide**

Pretreatment of the hypothalamic slices with IAP did not significantly alter the stimulation-evoked fractional release of [3H]norepinephrine (percent fractional release at S1 and S2 in control experiments, 0.583±0.021% and 0.568±0.023% of tissue tritium, respective-
TABLE 1. Effects of Calcitonin Gene-Related Peptide on Stimulation-Evoked and Basal Release of [3H]Norepinephrine in Rat Hypothalamic Slices

<table>
<thead>
<tr>
<th>Drugs added before S2 and b2</th>
<th>Fractional release (%)</th>
<th>Fractional release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1</td>
<td>S2</td>
</tr>
<tr>
<td>Control (n=8)</td>
<td>0.607±0.033</td>
<td>0.578±0.026</td>
</tr>
<tr>
<td>CGRP 1×10^{-8} M (n=8)</td>
<td>0.608±0.035</td>
<td>0.504±0.024</td>
</tr>
<tr>
<td>CGRP 1×10^{-8} M (n=8)</td>
<td>0.593±0.021</td>
<td>0.368±0.023*</td>
</tr>
<tr>
<td>CGRP 1×10^{-7} M (n=8)</td>
<td>0.612±0.026</td>
<td>0.362±0.031*</td>
</tr>
<tr>
<td>CGRP 3.3×10^{-7} M (n=8)</td>
<td>0.540±0.039</td>
<td>0.313±0.035*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. SI, first electrical stimulation; S2, second electrical stimulation; bl, prestimulation period before SI; b2, prestimulation period before S2; CGRP, calcitonin gene-related peptide.

Rat hypothalamic slices were stimulated twice (SI and S2) at 1 Hz (20 mA, unipolar rectangular pulses of 2-msec duration for 2 minutes). CGRP was added 14 minutes before S2. Fractional release at S1 and S2 was calculated by subtracting basal overflow (bl and b2) from total overflow of tritium during stimulation period (2-minute stimulation and the following 5 minutes) and is expressed as percentage of tissue tritium content at stimulation onset. CGRP effects on stimulation-evoked and basal release of tritium were expressed as S2/S1 and b2/bl ratios of tritium overflow, respectively.

*<p>0.05 compared with corresponding control.

The inhibitory action of CGRP on stimulation-evoked norepinephrine release was not significantly affected by IAP pretreatment (S2/S1 ratio: control in IAP-pretreated slices, 0.979±0.035, n=8, control in vehicle slices, 0.948±0.037, n=8; 1×10^{-8} M CGRP in IAP-pretreated slices, 0.602±0.050, n=8, in vehicle slices, 0.611±0.018, n=8; 1×10^{-7} M CGRP in IAP-pretreated slices, 0.533±0.043, n=8, in vehicle slices, 0.564±0.041, n=8).

Effects of Calcitonin Gene-Related Peptide in Combination With Bay K 8644 on Stimulation-Evoked Tritated Norepinephrine Release

Bay K 8644 (1×10^{-6} M) alone did not influence the stimulation-evoked release of [3H]norepinephrine. However, the inhibitory potency of CGRP on norepinephrine release was significantly attenuated in the presence of Bay K 8644 but not completely abolished (S2/S1 ratio: control, 0.959±0.050, n=8, 1×10^{-6} M Bay K 8644 alone, 0.921±0.034, n=8, 1×10^{-7} M CGRP alone, 0.598±0.036, n=8, p<0.05 compared with control; 1×10^{-7} M CGRP in combination with 1×10^{-6} M Bay K 8644, 0.850±0.016, n=8, p<0.05 compared with 1×10^{-7} M CGRP alone).

Discussion

In the present study, we investigated the effects of CGRP on [3H]norepinephrine release in the rat hypothalamus, a region in which both norepinephrine and CGRP are present in abundance. The results show that CGRP inhibited stimulation-evoked [3H]norepinephrine release in rat hypothalamic slices in a dose-dependent manner. The precise mechanisms responsible for the presynaptic effects of CGRP remain unclear. It has been shown that the release of norepinephrine in hypothalamus is highly dependent on presynaptic a2-adrenergic receptor functions. To determine whether or not the inhibitory action of CGRP on norepinephrine release might be mediated by a2-adrenergic receptors, we have studied its effects in combination with the a2-adrenergic receptor antagonist RX 781094. The blockade of a2-adrenergic receptors by RX 781094 caused no changes in the CGRP-induced reduction in stimulation-evoked [3H]norepinephrine release. Thus, it is considered unlikely that the inhibitory effects of CGRP on hypothalamic norepinephrine release might be mediated by the presynaptic a2-adrenergic receptors.

To explain the neuroinhibitory effects of CGRP, it could be proposed that CGRP might act in noradrenergic neurons by mobilizing one of the distinct molecular pathways probably involved in the mechanisms of norepinephrine release, i.e., cyclic adenosine monophosphate-mediated or Ca2+-mediated signal transductions. IAP inactivates the G, protein by ADP ribosylation, and this toxin has been widely used to determine the involvement of the G, protein in the receptor-mediated inhibition of adenylate cyclase. The present study demonstrates that pretreatment of hypothalamic slices with IAP showed no effects on the inhibitory effects of CGRP on stimulation-evoked [3H]norepinephrine release. The data may support the idea that the action of CGRP could not be mediated by the coupling of the receptor by means of the G, protein. In cardiac tissues, the CGRP effect is considered to be attributable to the activation of adenylate cyclase by stimulation of its specific receptor. However, Goltzman and Mitchell have shown that CGRP receptors in the brain and in the adrenal glands are not coupled to adenylate cyclase, which might be consistent with our present results.

On the other hand, it is generally accepted that Ca2+ is responsible for the neurotransmitter release from the nerve terminals and that an increase in intracellular Ca2+ concentration is the signal that initiates the secretion of neurotransmitters. This study shows that the inhibitory effect of CGRP on stimulation-evoked norepinephrine release from hypothalamic slices was significantly attenuated in the presence of Bay K 8644, a dihydropyridine-sensitive Ca2+ channel agonist, but not completely abolished. The results suggest that CGRP might partially interact with dihydropyridine-sensitive Ca2+ channels and modulate intracellular Ca2+ mobil-
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zation. Kline and Pang have observed that CGRP relaxed the rat tail artery contraction induced by noradrenaline in Ca\(^{2+}\)-free bathing medium and concluded that CGRP exerted its effect by inhibiting Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores. Recently, Nelson et al showed that CGRP hyperpolarized arterial smooth muscle cells by activating K\(^+\) channels on cell membranes, which indicated that CGRP might act as a K\(^+\) channel opener. Weston proposed that the effects of K\(^+\) channel openers were mediated partly by a hyperpolarization-induced closure of L-type plasmalemmal Ca\(^{2+}\) channels and further demonstrated that the K\(^+\) channel openers might decrease transmitter release and action potential duration and frequency in autonomic neurons. The inhibition of Ca\(^{2+}\) availability for the exocytosis of noradrenaline might partially explain the neuroinhibitory action of CGRP, although further studies are required to assess more properly the mechanisms of direct or indirect effects of CGRP on Ca\(^{2+}\) conductance in nervous tissues.

In summary, the results of the present study show that CGRP inhibited stimulation-evoked noradrenaline release in rat hypothalamus. The inhibition was partially reversed by the Ca\(^{2+}\) agonist Bay K 8644 but not by the \(\alpha\)-adrenergic antagonist or the G-protein inhibitor. These results suggest that the CGRP action might be mediated, at least in part, by interactions with dihydropyridine-sensitive Ca\(^{2+}\) channels and intracellular Ca\(^{2+}\) mobilization. Furthermore, the finding supports the hypothesis that CGRP may be involved in the regulation of sympathetic nervous activity in the central nervous system.

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