Modulation of Norepinephrine Release by Galanin in Rat Medulla Oblongata

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

Galanin, a 29-amino acid peptide, is widely distributed in both the central and peripheral nervous systems and is colocalized with catecholamines, although its physiological significance remains to be elucidated. In the present study we investigated the regulatory mechanisms of galanin on norepinephrine release in rat medulla oblongata. In slices of medulla oblongata of Sprague-Dawley rats, galanin inhibited the stimulation-evoked [3H]norepinephrine release in a concentration-dependent manner (fractional release ratio during electrical stimulation: control 0.937±0.043, mean±SEM, n=6; galanin 1×10⁻⁹ M 0.501±0.037, n=6, p=0.05; and galanin 1×10⁻⁸ M 0.299±0.018 n=6, p<0.05). Galanin potentiated inhibition of [3H]norepinephrine release by the a₁-agonists (UK 14,304 and clonidine). The blockade of a₁-adrrenergic receptors by RX 781094 diminished the inhibition of norepinephrine release by galanin. Pretreatment of pertussis toxin, which interferes with the coupling of inhibitory guanosine triphosphate-binding proteins to adenylyl cyclase, significantly attenuated the suppressive effects of galanin on norepinephrine release. In slices of medulla oblongata obtained from spontaneously hypertensive rats (SHR), the inhibitory effect of galanin on norepinephrine release was significantly less than in those from age-matched Wistar-Kyoto rats. These results show that galanin might inhibit the stimulation-evoked norepinephrine release in rat medulla oblongata, at least partially mediated by a₁-adrenergic receptors and the pertussis toxin-sensitive guanosine triphosphate-binding proteins. Moreover, less suppression of norepinephrine release by galanin in SHR suggests that galanin might be involved in the regulation of central sympathetic nervous activity in hypertension. (Hypertension 1992;20:361–366)

KEY WORDS • peptides • medulla oblongata • norepinephrine • receptors, adrenergic, a₁ • guanosine triphosphate • pertussis toxins • Wistar-Kyoto rats • spontaneously hypertensive rats

Galanin is a biologically active neuropeptide composed of 29 amino acids that was isolated from porcine upper intestine.¹ The galanin-like immunoreactivity in the brain is demonstrated not only in pigs, but also in rats, monkeys, and humans.²⁻³ Immunohistochemical studies have shown that galanin-immunoreactive neurons are present in the brain and spinal cord as well as in neuronal structures in several peripheral systems.² Skofitsch and Jacobowitz⁴ have observed the quantitative distribution of galanin-like immunoreactivity in rat central nervous system and reported that high concentrations were determined in the median eminence, hypothalamus, locus coeruleus, medulla oblongata, and the caudal spinal trigeminal nucleus.

Recent evidence has suggested that galanin might actively participate in the central control of blood pressure and other cardiovascular functions because a high concentration of galanin has been found in the dorsal cardiovascular centers, particularly in the nucleus tractus solitarii of rat medulla oblongata.⁴ Häfström et al⁵ have observed that intracisternally injected galanin in the nanomolar range induced a significant hypotension in anesthetized rats and further reported that the combined treatment of galanin with neuropeptide Y resulted in a more prolonged hypotensive action.

In several areas, galanin has been shown to coexist with other peptides or amines, such as γ-aminobutyric acid, norepinephrine, dopamine, serotonin, and acetylcholine.²⁻⁶⁻⁹ In many cases, the presence of coexisting peptides is believed to influence the release of classic neurotransmitters, although little is understood about the interactions between galanin and these transmitters at either presynaptic and postsynaptic sites. Nordström et al⁶ have reported that galanin significantly inhibits dopamine release from the rat median eminence and proposed that this peptide could act as a dopaminergic neuromodulator in this region. Fisone et al⁷ have found that galanin inhibits acetylcholine release in the ventral hippocampus of the rat both in vivo and in vitro. Recently, our colleagues have demonstrated that noradrenergic neurons containing galanin in locus coeruleus preferentially project to the hypothalamus, cerebral cortex, brain stem, and spinal cord of rats.⁸ Additionally, we have reported that galanin reduced the

From the Division of Cardiology (K.T., I.N., Y.M.), Department of Medicine and the Third Department of Medicine (S.T.), Wakayama Medical College, Wakayama, Japan, and Neurochemistry Research Laboratories (M.G.), New York University Medical Center, New York.


Address for correspondence: Kazushi Tsuda, MD, Division of Cardiology, Department of Medicine, Wakayama Medical College, 27,7-Bancho, Wakayama 640, Japan.

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Animals
Galanin was donated by Dr. David Schlesinger (Cell KG, Ingelheim, Germany, respectively. The α2-antagonist 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline (UK 14,304) and clonidine were received from RPI Corp., Mt. Prospect, IL. The a2-antagonist 2-
[2-(1,4-benzodiazepanyl]-2-imidazolin HCl (RX 781094) was received from RPI Corp., Mt. Prospect, Ill.

The presence of a high density of galanin in the nucleus tractus solitarii may suggest the idea that the peptide has an inhibitory action on catecholamine release in this region and has a significant role in cardiovascular regulations. In the present study, to gain further insight into the regulatory mechanisms of galanin on central sympathetic nervous activity, we investigated the influences of galanin on norepinephrine release in rat medulla oblongata and further examined the effects of the α2-adrenergic agonist and antagonist as well as the effects of inactivation of the G protein by pertussis toxin on the modulation of norepinephrine release in this region. In the second series of the experiments to test the possibility of abnormal peptidergic regulation of central norepinephrine release in hypertension, we studied whether galanin-mediated regulation of norepinephrine release might be altered in the medulla oblongata of spontaneously hypertensive rats (SHR).

Methods

Animals
Male Sprague-Dawley (SD) rats (weight, 200–250 g) from Taconic Farms, Germantown, N.Y., were used for the fundamental investigation of the effects of galanin in rat medulla oblongata. Male SHR (9–10 weeks old; Taconic Farms) were studied in comparison with age-matched male Wistar-Kyoto (WKY) rats (Taconic Farms). The body weight of the SHR was 197.5±2.0 g (n=6) and 177.3±3.5 mm Hg in SHR rats (n=6) and 114.7±6.2 mm Hg in WKY rats (n=6).

Drugs
The α2-agonist 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline (UK 14,304) and clonidine were received from Pfizer Inc., New York, and Boehringer Ingelheim KG, Ingelheim, Germany, respectively. The α2-antagonist 2-
[2-(1,4-benzodiazepanyl]-2-imidazolin HCl (RX 781094) was received from RPI Corp., Mt. Prospect, Ill. Galanin was donated by Dr. David Schlesinger (Cell Biology and Kaplan Cancer Center, New York University Medical Center, New York). Purified pertussin toxin (islet activating protein) was purchased from List Biological Laboratories Inc., Campbell, Calif. All other drugs used were standard laboratory reagents of analytical grade.

Experimental Procedure
The rats were decapitated, and the whole medulla oblongata was rapidly dissected on ice according to the method described previously. The frontal section was cut from the level of the nucleus nervi facialis to the pyramidal decussation. The isolated medulla oblongata was sliced at 0.3-mm thickness with 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 Krebs-Ringer bicarbonate buffer (in mM: NaCl 118.0, KCl 4.80, CaCl2 1.20, KH2PO4 1.15, MgSO4 1.20, NaHCO3 25.0, glucose 11.1, ascorbic acid 0.11, and disodium EDTA 0.04 saturated with a 95% O2-5% CO2 mixture at 37°C, pH 7.4). The slices were incubated with 6 ml Krebs-Ringer bicarbonate 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 (5–6 slices) were rinsed with fresh buffer, they were transferred to a superfusion chamber (volume 200 μl), jacketed with 37°C water and suspended between two platinum electrodes (25 mm apart, 2 mm long). The slices were continuously superfused with Krebs-Ringer bicarbonate buffer at a rate of 0.7 ml/min. The superfusate was collected 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 (at 130 minutes). For electrical stimulation, trains of unipolar and rectangular pulses (1 Hz, 20 mA, 2-msec duration for 2 minutes) were delivered with a stimulator (model S4K, Grass Instrument Co., Quincy, Mass.). The electrical stimulation was applied at 67 minutes (S1) and 116 minutes (S2) after the beginning of the superfusion. At the end of the experiment, the slices were solubilized by sonication for 20 seconds. Radioactivity in the collected samples and solubilized tissues was determined by liquid scintillation spectrometry (Packard Tri-carb Liquid Scintillation Spectrometer, model 3255, Packard Instrument Co., Sterling, Va.).

The amount of tritium released 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 the sample collection (the tritium released into superfusate after that point plus the tritium remaining in the tissue at the end of the experiment) and was expressed as a percentage of fractional release. Basal overflow during the 2 prestimulation periods (b1 and b2, respectively) was evaluated from the tritium collected in the two 7-minute samples just before S1 and S2. The overflow of tritium evoked by nerve stimulation was calculated by subtracting the basal overflow during the 7-minute prestimulation period from the value in samples collected during the 2-minute stimulation period and 5 minutes after the electrical stimulation (total 7 minutes). The tritium content of the first fraction collected ranged consistently from 5,000 to 7,000 disintegrations per minute and the tritium remaining in the
to the superfusion medium 28 minutes before SI and maintain until the end of the experiment. The superfusion medium in the control experiments, SI and S2 were performed in the absence of any added drugs. The effects of galanin, UK 14,304, and clonidine were evaluated only in S2, with SI serving as an internal control. Supervision with galanin, UK 14,304, and clonidine was initiated 14 minutes before S2. Fractional release during SI and S2 were calculated by subtracting basal outflow from the total outflow of tritium during stimulation period (2-minute stimulation and after 5 minutes) and is expressed as percentage of the tritium content of the tissue at the onset of stimulation. S1, first electrical stimulation; S2, second electrical stimulation; b1, prestimulation period before S1; b2, prestimulation period before S2; S2/S1, fractional release ratio during S2 and S1; b2/b1, fractional release ratio during b2 and b1. Data are represented as mean±SEM.

* p<0.05 compared with the control treatment and the corresponding controls were determined by one-way analysis of variance (ANOVA). To compare the means of the different study groups, the Wilcoxon rank-sum test was used. To examine the differences between SHR and WKY rats, statistical analyses were performed with the two-way ANOVA. A value of p<0.05 was accepted as the level of significance.

Results

Effects of Galanin Alone and in Combination With UK 14,304, Clonidine, and RX 781094 on the Tritiated Norepinephrine Release in Medulla Oblongata of Sprague-Dawley Rats

In the control experiments, the stimulation-evoked [3H]norepinephrine release in S1 and S2 does not differ significantly (S2/S1 ratio, 0.937±0.043, n=6). Table 1 shows the effects of galanin on the release of [3H]norepinephrine in slices of medulla oblongata of SD rats. Galanin strongly inhibited the stimulation-evoked [3H]norepinephrine release in a concentration-dependent manner (IC50 value, 1.5±0.4x10^-7 M, n=6), although the basal release of [3H]norepinephrine was not changed by these concentrations of the peptide.

To evaluate whether α2-adrenergic receptors are associated with the inhibitory action of galanin, we studied the effects of galanin in combination with UK 14,304, clonidine, and RX 781094, and pertussis toxin on the [3H]norepinephrine release in SD rats. In the control experiment, S1 and S2 were performed in the absence of any added drugs. The effects of galanin, UK 14,304, and clonidine were evaluated only in S2, with S1 serving as an internal control. Supervision with galanin, UK 14,304, and clonidine was initiated 14 minutes before S2 and maintained until the end of the experiment. The effects of the drugs on the stimulation-evoked [3H]norepinephrine release were determined by comparing the S2/S1 ratios (fractional release ratios during S2 and S1) obtained in control slices with the values in slices treated with the tested drugs in S2. Control superfusion chambers and chambers with several concentrations of the drugs were run in parallel.

To examine the effects of blockade of α2-adrenergic receptors, RX 781094 (1x10^-8 M) was added to the superfusion medium 28 minutes before S1 and maintained until the end of the experiment. To inactivate the G protein, the slices of medulla oblongata were preincubated for 1 hour at 37°C in 95% O2-5% CO2 atmosphere in a mixture of 1,380 µl Krebs-Ringer bicarbonate buffer and 120 µl 0.01 M sodium phosphate buffer that contained 0.05 M NaCl and 12 µg pertussis toxin (concentration 8 µg/ml). In the control experiment, the slices were incubated in the same buffer mixture without pertussis toxin.

In the second series of the experiment, the effects of galanin on the stimulation-evoked release of [3H]norepinephrine were examined in the slices of medulla oblongata of SHR as compared with WKY rats.

Statistics

Values are expressed as mean±SEM. Differences between the means of the drug treatment and their corresponding controls were determined by one-way analysis of variance (ANOVA). To compare the means of the different study groups, the Wilcoxon rank-sum test was used. To examine the differences between SHR and WKY rats, statistical analyses were performed with the two-way ANOVA. A value of p<0.05 was accepted as the level of significance.
FIGURE 1. Line graph shows effects of galanin (Gal) on stimulation-evoked [3H]norepinephrine release in the presence of an α2-adrenergic receptor antagonist, RX 781094 (10⁻⁸ M). RX 781094 was added to the superfusion medium 28 minutes before SI and maintained until the end of the experiment. Values are mean±SEM.

slices to RX 781094 before SI increased the stimulation-evoked [3H]norepinephrine release (fractional release during SI, 1.705±0.049%, n=6; during S2, 1.646±0.053%, n=6; S2/S1 ratio, 0.947±0.010, n=6). The data in Figure 1 show that the inhibition by galanin was significantly attenuated in the presence of RX 781094.

Effects of Pertussis Toxin on the Inhibition of Tritiated Norepinephrine Release by Galanin and UK 14,304 in Medulla Oblongata of Sprague-Dawley Rats

The α2-adrenergic receptors are negatively linked to adenylate cyclase via the G protein. We therefore examined whether the inactivation of the G protein by pertussis toxin may alter the inhibitory action of galanin on the stimulation-evoked [3H]norepinephrine release. The fractional release of [3H]norepinephrine release during electrical stimulation was not changed by the treatment of pertussis toxin. However, the inhibitory effect of galanin on [3H]norepinephrine release was significantly attenuated in slices pretreated with pertussis toxin (Table 2). Similarly, the inhibitory action of UK 14,304 on [3H]norepinephrine release was also reduced in the pertussis toxin–treated slices (Table 2).

Effects of Galanin on Tritiated Norepinephrine Release in Medulla Oblongata of Spontaneously Hypertensive Rats and Wistar-Kyoto Rats

The stimulation-evoked [3H]norepinephrine release from slices of medulla oblongata was not significantly different between SHR and WKY rats (percent fractional release during S1: SHR, 1.239±0.033% of total tissue radioactivity, n=6; WKY, 1.396±0.063%, n=6). The basal release of tritium also did not differ between SHR and WKY rats (percent fractional release during bl: SHR, 2.798±0.034% of total tissue radioactivity, n=6; WKY, 2.661±0.029%, n=6).

As shown in Figure 2, galanin significantly reduced the stimulation-evoked [3H]norepinephrine release both in SHR and WKY rats. The suppression by galanin was significantly more attenuated in SHR than in WKY rats (S2/S1 ratio: galanin 1×10⁻⁷ M: SHR, 0.883±0.050, n=6; WKY, 0.534±0.010, p<0.05; galanin 1×10⁻⁶ M: SHR, 0.542±0.013, n=6; WKY, 0.217±0.019, n=6, p<0.05).

Discussion

Galanin is colocalized with classic neurotransmitters such as norepinephrine, dopamine, or acetylcholine in specific neuronal systems in the brain.2-7-9 We therefore investigated the effects of galanin on norepinephrine release and its interactions with α2-adrenergic receptors in rat medulla oblongata. The results of the present study demonstrate that galanin inhibited the stimula-

### Table 2. Effects of Pertussis Toxin on Galanin-Induced and UK 14,304-Induced Reduction in Stimulation (1 Hz)-Evoked [3H]Norepinephrine Release in Medulla Oblongata of Sprague-Dawley Rats

<table>
<thead>
<tr>
<th>Drugs added before S2</th>
<th>S1</th>
<th>S2</th>
<th>S2/S1</th>
<th>b2/b1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pertussis toxin (+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=6)</td>
<td>1.338±0.103</td>
<td>1.298±0.127</td>
<td>0.967±0.031</td>
<td>0.782±0.033</td>
</tr>
<tr>
<td>Gal 1×10⁻⁷ M (n=6)</td>
<td>1.348±0.027</td>
<td>1.132±0.031*</td>
<td>0.841±0.035*</td>
<td>0.817±0.025</td>
</tr>
<tr>
<td>Gal 1×10⁻⁸ M (n=6)</td>
<td>1.372±0.085</td>
<td>0.779±0.053†</td>
<td>0.572±0.023†</td>
<td>0.812±0.020</td>
</tr>
<tr>
<td>UK 1×10⁻⁸ M (n=6)</td>
<td>1.200±0.049</td>
<td>0.578±0.029†</td>
<td>0.497±0.040†</td>
<td>0.809±0.011</td>
</tr>
</tbody>
</table>

| Pertussis toxin (−)   |      |      |       |       |
| Control (n=6)         | 1.328±0.031 | 1.245±0.032 | 0.937±0.036 | 0.799±0.010 |
| Gal 1×10⁻⁷ M (n=6)    | 1.259±0.040 | 0.641±0.057† | 0.507±0.040† | 0.806±0.011 |
| Gal 1×10⁻⁸ M (n=6)    | 1.357±0.047 | 0.446±0.027† | 0.334±0.024† | 0.815±0.016 |
| UK 1×10⁻⁸ M (n=6)     | 1.249±0.034 | 0.255±0.044† | 0.184±0.038† | 0.815±0.021 |

Slices were pretreated with pertussis toxin (8 μg/ml), incubated with [3H]norepinephrine, and superfused as described in text. Effects of galanin (Gal) and UK 14,304 (UK) were expressed as S2/S1 ratios of tritium overflow evoked by the two stimulation periods. S1, first electrical stimulation; S2, second electrical stimulation; b1, prestimulation period before S1; b2, prestimulation period before S2; S2/S1, fractional release ratio during S2 and S1; b2/b1, fractional release ratio during b2 and b1. Values are mean±SEM.

*P<0.05 compared with the experiments in the presence of same concentrations of galanin or UK 14,304 alone.
†P<0.05 compared with the corresponding control.
activation of α2-adrenergic receptors. Additionally, the blockade of α2-adrenergic receptors by RX 781094 diminished the inhibitory effects of galanin on norepinephrine release, which would support the hypothesis that galanin can act on α2-adrenergic receptors in rat medulla oblongata. The possibility that galanin interacts with α2-adrenergic receptors was further suggested by the evidence that galanin-induced feeding behavior in rats depended specifically on functional α2-receptor sites. It was also shown that the effects of galanin on the release of luteinizing hormone releasing hormone in rat brain was blocked by phentolamine.

The signal transduction system via the α2-adrenergic receptors is believed to be linked to the G protein of the membranes. Pertussis toxin inactivates the G protein by ADP ribosylation of the α subunit and has been used to determine the involvement of the G protein in the receptor-mediated inhibition of adenylate cyclase. The present study shows that galanin-induced decrease in [3H]norepinephrine release was significantly attenuated in the slices pretreated with pertussis toxin. This finding indicates that the inhibitory modulation of [3H]norepinephrine release by galanin might be, in part, mediated by the coupling of the receptor by means of the G protein. Nishibori et al reported that galanin inhibited the accumulation of cyclic adenosine monophosphate content in rat cerebral cortex. In receptor binding studies, it was shown that 125I-galanin binding sites in rat brain were affected by GTP and its analogues and by pertussis toxin-catalyzed ADP-ribosylation, which suggests that galanin receptor is coupled to an inhibitory G protein in the central nervous system. However, it remains to be elucidated whether galanin can interact with only the α2-adrenergic receptor–mediated G protein or with other coupling proteins.

Our results also showed that the inhibition of norepinephrine release by galanin was significantly attenuated in the medulla oblongata of SHR compared with age-matched WKY rats. The mechanisms responsible for impaired suppression of norepinephrine release by galanin are still uncertain. It has been reported that there was a specific decrease in the density of [3H]clonidine binding sites or [3H]yohimbine binding sites in the medulla oblongata of SHR. In agreement with these previous observations, we have reported that the α2-agonist (UK 14,304)–induced inhibition of stimulation-evoked [3H]norepinephrine release was significantly less in the slices of medulla oblongata from SHR than in those from WKY rats. Thus, less inhibitory effects of galanin on norepinephrine release can be partially explained by the finding that the α2-adrenergic receptor function is decreased in the medulla oblongata of SHR, although further studies are required to assess properly the interactions of galanin with α2-adrenergic receptors and their role in the regulation of norepinephrine release in the central nervous system of SHR.

It has been shown that galanin-like immunoreactivity-containing cell bodies in the brain also contain immunoreactivity of substance P or calcitonin gene-related peptide. Recently, alterations in regional contents of peptide hormones such as calcitonin gene-related peptide or neuropeptide Y have been demonstrated in the brain of SHR compared with WKY rats, although there are no studies evaluating whether the galanin content might be changed in central nervous system of SHR.
Goldstein M, Deutch AY: The inhibitory actions of NPY and sympathetic tone in hypertension. It would be possible that the quantitative abnormality might cause less sensitivity to exogenously applied galanin in medulla oblongata of SHR.

In summary, the results of the present study demonstrate that galanin inhibited stimulation-evoked norepinephrine release in rat medulla oblongata, and that a part of the mechanisms can be explained by the interactions with presynaptic α2-adrenergic receptors and the pertussis toxin-sensitive GTP-binding proteins in this region. Although the precise role of galanin in the pathogenesis of hypertension is still uncertain, the impaired modulation of norepinephrine release by galanin in medulla oblongata of SHR suggests the possible involvement of the peptide in the regulation of central sympathetic tone in hypertension.

References
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