Neuronal expression of c-Fos protein in the brain after intraperitoneal injection of galanin in Wistar rats

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(Received September 30, 2006; Accepted December 11, 2006)

Abstract: Galanin has been known as a biological active neuropeptide originally isolated from porcine intestine, and it has been known to be involved in various physiological and behavioral actions. As for the gastrointestinal functions galanin inhibits gastric acid secretion. The present study was aimed to examine the distribution of c-Fos protein in the brain after intraperitoneal injection of galanin in Wistar rats. In the test experiments, one and a half hours after the intraperitoneal injection of galanin, rats were perfused transcardially with saline solution and fixed with fixatives. Then brains were removed and sectioned at 40 μm in thickness. Every fourth section was treated with polyclonal anti-c-Fos antiserum, and c-Fos protein was immunohistochemically stained using the avidin-biotin complex method. In the control experiments saline solution was intraperitoneally injected, and brains were removed and processed similarly as described above. Intraperitoneal injection of galanin induced much more c-Fos-immunoreactive (c-Fos-ir) neurons in the area postrema (AP) in the test experiment than in the control experiment. The present results suggest that galanin may have central actions through the AP neurons in playing roles in the gastroenteric functions.

Key words: Stomach; Galanin; Gastroenteric function; c-Fos; Neurons; Central nervous system;

INTRODUCTION

Galanin is a 29-amino acid residue neuropeptide, originally isolated from porcine intestine in 1983 by Tatemoto and coworkers¹. Galanin has been known as a biological active peptide that shows many physiological and behavioral actions such as learning and memory, epileptic activity, nociception, spinal reflexes and feeding²,³,⁴,⁵. It was reported that galanin increased food intake after its central injection, and the activity of galanin was found to be induced via its binding to galanin receptor in the rat hypothalamus⁶. Recently, it was reported that the intracerebroventricular injection of galanin induced c-fos in hypothalamus as well as some other brain regions⁷. On the other hands the physiological actions of peripheral administered galanin were also reported including the contraction of smooth muscle⁸, activation of an ATP-dependent potassium channel⁹, and inhibition of glucose-stimulated insulin release¹⁰. It was also reported that galanin decreased secretion of gastric acid¹¹,¹². However, whether the effects of peripheral administration of galanin were due to be via the central neurons has not been clarified. Thus in this study it was examined whether the peripheral...
administration of galanin may induce c-Fos protein in the brain. c-Fos expression method presented evidence that proto-oncogene \( \textit{c-fos} \) expresses c-Fos protein rapidly and transiently within neurons in response to synaptic activation\(^{13,14,15}\). That is, the expression of c-Fos protein in neurons indicated the excitation of neurons in response to synaptic activation after some stimulations. In the present study we undertook to identify the neuronal expression of c-Fos protein throughout the CNS that might be produced after intraperitoneal administration of galanin. We found that c-Fos protein was expressed in many neurons in several nuclei throughout the brain.

**MATERIALS AND METHODS**

We undertook to examine which neurons in the CNS may express c-Fos protein after intraperitoneal injection of galanin. In the test experiments male Wistar rats weighing 230–250 g (\( n=4 \)) were intraperitoneally injected with galanin (250 \( \mu \)g/kg) in 250 \( \mu \)l of 0.9% NaCl solution. One and a half hours after the injection of galanin, rats were anesthetized with pentobarbital sodium (intraperitoneally, 50 mg/kg), and perfused via the left ventricle with about 20 ml of saline to flush out the blood. This was immediately followed by 100 ml of 0.5% glutaraldehyde and 4% paraformaldehyde (PFA, Merck, German) in 0.1 M phosphate buffer (PB, pH 7.4), and 400 ml of 4% PFA in PB. The brains were removed and cut into two blocks at the level between superior and inferior colliculus. They were fixed in 4% PFA in PB for further 1.5 h at 4\( ^\circ \)C, then soaked stepwise in 10, 20, 25% sucrose in PB at 4\( ^\circ \)C. The brains were frozen and cut into serial transverse sections at 40 \( \mu \)m in thickness. The sections were collected in plates containing PB chilled by ice water. One group of every fourth section was rinsed in 0.1 M Tris-saline (TS, pH 7.4) three times, incubated with 0.5% bovine serum albumin (BSA) in TS for 20 min and incubated with sheep anti-c-Fos polyclonal antiserum (1:100; \textit{sc-52}, Santa Cruz Biotech. Inc., USA) in 0.5% BSA at 4\( ^\circ \)C for 16 h. Afterwards, the sections were rinsed three times in TS, and then incubated in avidin and biotin solution respectively for 15 min. Thereafter they were incubated for 20 min with 2% normal rabbit serum in TS to block nonspecific bindings, and incubated for 60 min with anti-sheep IgG in TS. After rinsed three times in TS, the sections were incubated for 60 min in Vectastatin ABC kit (Vector Lab., Burlingame, CA, USA), and then treated with diaminobenzidine-nickel solution containing 0.003% \( \text{H}_2\text{O}_2 \). After that, the sections were rinsed in TS twice and rinsed further in PB. They were mounted on glass slides and dried at room temperature. The sections on the slides were dehydrated in a graded ethanol series (50%, 70% 95%, 99%), infiltrated with xylene and coverslipped in Permount (Fisher Comp., USA). Control rats (\( n=4 \)) were sham-operated and injected with 250 \( \mu \)l of 0.9% NaCl solution. Brain sections were similarly processed as above. c-Fos protein localized in neuronal nuclei was visualized as black precipitates of nickel-intensified diaminobenzidine reaction products. c-Fos-immunoreactive (c-Fos-ir) neurons were surveyed under bright-field microscopy. Brain histology was checked against the rat brain atlas of Swanson\(^{16}\). Mann-Whitney \( U \)-tests were performed to compare the data between the two groups.

**RESULTS AND DISCUSSION**

Intraperitoneal injection of galanin (250 \( \mu \)g/kg) induced c-Fos protein in central nuclei and circumventricular organs. Fig.1 shows c-Fos-ir neurons found in the area postrema (AP). Fig.2 and Fig.3 show histograms of c-Fos-ir neurons in the nuclei and the circumventricular organs in the brain. The c-Fos-ir neurons induced in each nuclei were averaged per one brain section. Increases in the number of c-Fos-ir neuronal profiles, compared to that in control experiment, were observed in nuclei and circumventricular organs from the medulla oblongata to the basal telencephalon after intraperitoneally injected galanin.

**Fig. 1.** The photomicrographs of c-Fos-immunoreactivity in the area postrema (a). c-Fos-ir neurons were stained as black dots indicated by arrows in the enlarged microphotograph (b). Scale bars: 0.5 mm.
injection of galanin. The site in which significantly much more neurons expressed c-Fos protein in the test experiments than in the control experiments was the AP (38 ± 14 c-Fos-ir neurons in the test, 12 ± 2 number of c-Fos-ir neurons in the control, 8 ± 3 c-Fos-ir neurons. c-Fos protein was also detected in many other nuclei such as the dorsal motor nucleus of the vagus nerve (DMX), solitary tract nucleus (NTS), ventromedial hypothalamic nucleus (VLM), locus coeruleus nucleus (LC), lateral parabrachial nucleus (Pbl), paraventricular nucleus thalamus (PVT), arcuate nucleus hypothalamus (ARH), dorsal medial thalamus nucleus (DMH), central nucleus amygdala (CEA), posterior hypothalamic nucleus (PVP), anterior hypothalamic nucleus (AHP), supraoptic nucleus (SO), retrochiasmatic area (RCh), subfornical organ (SFO). However, in these central nuclei or the circumventricular organs no significant differences in the c-Fos-ir neurons between the test and control experiments were observed.

It is to be considered that the diffusion of galanin into the brain is limited by the blood-brain barrier (BBB) mechanism. However, the BBB is lacking in the circumventricular organs so that peptides or peptide fragments might possibly penetrate into the brain\(^{17}\). Thus, galanin may possibly penetrate the brain. That is why we tried peripheral injection of galanin to observe the expression of c-Fos protein in central neurons. In this study intraperitoneal injection of galanin induced expression of c-Fos protein in many neurons in the AP. The AP is known to be one of the circumventricular organs which lie outside the blood-brain barrier. The AP/NTS, the Pbl, the CEA and the PVH are brain sites that contain galanin-immunoreactive cell bodies and terminals\(^{1,18,19,20,21}\). These brain regions are involved in the regulation of feeding, and injection of galanin into the AP/NTS and above-mentioned brain regions stimulate food intake\(^{22}\). As described above AP is known to be lack in the blood brain barrier and galanin might stimulate directly the galanin receptors, though the type of the galanin receptor has to be identified out of three receptors GalR1, GalR2, GalR3 and receptor subtypes, gal1, gal2 and gal3\(^{23,24,25,26}\). According to the results by Mariann et al, galanin might stimulate neurons in the AP/NTS via galanin receptors\(^{27,28,29}\). Koegler and Ritter reported that galanin injection into the AP/NTS stimulate feeding in rats, and suggested that galanin receptors in the AP/NTS region mediate feeding in response to galanin and that the galaninergic nerve terminals innervating these receptors may originate in part from cell bodies in the PVH\(^{22}\). It is conceivable that intraperitoneal injection of galanin induced expression of c-Fos protein in not so many neurons in the central nuclei, because galanin usually causes inhibitory effect rather than stimulatory effect on various physiological functions\(^{1,12,17,18,26,27,30,31}\). It is also conceivable that it might be more difficult for galanin to
stimulate central neurons directly. In this study, it was clarified that galanin may stimulate central neurons via the neurons in the AP, suggesting playing some physiological functions through the central nervous system.

REFERENCES
22) Koegler FH, Ritter S. Galanin injection into the nucleus of the solitary tract stimulates feeding in


