Exogenous galanin regulates capsaicin-evoked substance P release from primary cultured dorsal root ganglion neurons

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Abstract

OBJECTIVES: To determine the effect of exogenous galanin on capsaicin-evoked substance P release from primary cultured embryonic rat dorsal root ganglion (DRG) neurons.

METHODS: DRG was dissected out from embryonic 15-day-old Wistar rat and cultured as dissociated cells for 2 days then exposed to galanin (1 nmol/L, 10 nmol/L, 100 nmol/L). After 4 days incubation with exogenous galanin, the levels of mRNAs for SP and vanilloid receptor 1 (VR1) and protein for VR1 were estimated by RT-PCR and Western blot, respectively. Basal SP release and capsaicin-evoked SP release levels were measured by radioimmunoassay (RIA).

RESULTS: The amount of VR1 mRNA and VR1 protein expression and capsaicin-evoked SP release in cultured DRG neurons increased significantly after incubation with exogenous galanin compared with control DRG neurons at the same time point, whereas the amount of SP mRNA and basal SP release were not affected after incubation with exogenous galanin.

DISCUSSION: Exogenous galanin may promote capsaicin-evoked SP release from primary cultured DRG neurons. The elevation of the levels of VR1 mRNA and VR1 protein induced by exogenous galanin implicated that VR1 may be involved in the mechanisms of SP release evoked by capsaicin.

INTRODUCTION

Galanin, a 29-amino-acid neuropeptide in most species (30-amino-acid in human), is widely distributed throughout the nervous system including dorsal root ganglion (DRG) neurons and is involved in the regulation of nociceptive functions [4, 9, 11, 17, 22, 23]. The actions of galanin are mediated by three receptor subtypes (GalR1, GalR2, and GalR3), which are coupled to different intracellular effector systems [4, 7, 9, 19]. It has been suggested that galanin may exert a predominantly inhibitory action on nociceptive and neuropathic pain in the spinal cord mediated by activation of GalR1 or GalR3 [3, 17]. However, the spinal pronociceptive effect of galanin could be mediated by activation of spinal GalR2 [6, 16]. Interestingly, GalR2 and vanilloid receptor 1 (VR1) are co-expressed in DRG neurons suggesting that galanin-induced effects are mediated by GalR2 on capsaicin-sensitive primary sensory neurons and galanin can modulate VR1...
function [10]. VR1, a non-selective cation channel, is a molecular sensor for detecting adverse stimuli and a key element for inflammatory nociception [13]. VR1 can be activated by capsaicin, the main ingredient in hot chili peppers, which elicits a sensation of burning pain by selectively activating sensory neurons which release sensory neuropeptides such as substance P (SP) from its peripheral nerve endings [24, 25]. SP, an 11-amino-acid neuropeptide, is found in sensory nerves innervating peripheral tissues [21]. Release of SP from peripheral endings causes a series of local inflammatory responses referred to as neurogenic inflammation [26]. Interestingly, systemic or topical administration of capsaicin has been shown to up-regulate galanin and to down regulate SP in primary sensory neurons [1]. Although there have been significant advances in understanding the role of galanin in nociceptive and neuropathic pain, little is known concerning the regulatory actions of galanin on SP expression or release. Studies were performed using primary cultured embryonic rat DRG neurons to determine exogenous galanin at different concentrations (1 nmol/L, 10 nmol/L, 100 nmol/L, respectively) on SP and VR1 expression and capsaicin-evoked SP release.

MATERIALS AND METHODS

DRG cell culture preparations

DRG was dissected out from embryonic 15-day-old Wistar rats. The animals were obtained from the Experimental Animal Center of Shandong University of China. All animals were cared for in compliance with the Guide for the Care and Use of Laboratory Animals (revised 1996; http://www.nap.edu). All procedures described herein were reviewed by and had prior approval from the local animal ethics committee. DRG prior to establishment in culture was digested with 0.25% trypsin (Sigma) in D-Hanks solution at 37°C for 10 minutes and centrifuged for 5 minutes at 1x10³ rpm. The supernatants were removed and the pellets were resuspended in Dulbecco's Modified Eagle Medium (Gibco) and triturated using a sterile modified Pasteur's glass pipette. Cells were then filtered using a 130 μm filter followed by counting. Dissociated DRG cells were then cultured in media (DMEM/F-12) with 5% CO₂ for 24 hours and then maintained in culture media containing cytarabine (ara-C) (5 μg/ml) for another 24 hours to inhibit growth of non-neuronal cells, and then cultured in culture media for another 4 days with media change every 2 days. The composition of the culture media is D-MEM/F-12 (1:1) supplemented with 5% fetal bovine serum, 2% B-27 supplement (Gibco), insulin (0.25 μg/ml, Sigma), L-glutamine (0.1 mg/ml, Sigma), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Expression of exogenous galanin on DRG neurons

To determine whether exogenous galanin influences SP release in DRG cultures, exogenous galanin (1 nmol/L, 10 nmol/L, 100 nmol/L, respectively) was added to the culture media at 2 days of culture age. These cultures were then incubated for additional 4 days with media change every 2 days. The culture media contained exogenous galanin (1 nmol/L, 10 nmol/L, 100 nmol/L, respectively) during the 4 days incubation. DRG neurons were cultured continuously in culture media for 6 days as control.

RNA extraction and RT-PCR

The mRNA levels of SP and VR1 were analyzed by RT-PCR after 4 days incubation with exogenous galanin at different concentrations. The expression of β-actin was also determined as an internal control. Total DRG cell RNA of each flask was isolated by TRizol. cDNA synthesis was performed with M-MLV reverse transcriptase. The gene-specific primers were synthesized by use of the published cDNA sequences for SP, VR1, and β-actin. The synthetic oligonucleotide primer sequences for SP, VR1, and β-actin are as follows:

- SP 5’-GCC CTT TGA GCA TCT TCT TC-3’ (upper primer) and 5’-GTC TGA GGA GGT CAC CAC AT-3’ (lower primer).
- VR1 5’-CTG ACG GCA AGG ATG ACT-3’ (upper primer) and 5’-CCT AAG CAG ACC ACC CAA-3’ (lower primer).
- β-actin 5’-ATC ATG TTT GAG ACC TTC AAC-3’ (upper primer) and 5’-CAT CTC TTG CTC GAA GTC CA-3’ (lower primer).

The predicted size of the amplified SP, VR1, and β-actin DNA products were 450 bp, 372 bp, and 317 bp, respectively.

PCR amplification was performed for 35 cycles. The cycle profile included denaturation for 45 s at 94°C, annealing for 45 s at 58°C, and extension for 60 s at 72°C. PCR was performed within the range that demonstrates a linear correlation between the amount of cDNA and the yield of PCR products.

The amplified products were analyzed by standard agarose gel electrophoresis and stained with ethidium bromide, visualized by a UV transilluminator and photographed. The photographs were scanned and the electrophoresis gel images were analyzed quantitatively by using a Totallab image analysis software. The levels of SP mRNA and VR1 mRNA were expressed as the ratio of the gene to β-actin.
Western blot analysis for VR1 proteins expression

VR1 protein expression was analyzed by Western blot. Fresh cultured DRG neurons 4 days later after galanin incubation were homogenized in 10 mmol/L Tris-homogenization buffer (pH 7.4) with protease inhibitors (Sigma). The samples were centrifuged at 12,000 rpm for 20 minutes and the supernatant collected for Western blot. After determining the protein concentrations of the supernatants (BSA method, standard: BSA), 50 μg protein of each sample was loaded onto the 8% SDS gel, separated by electrophoresis and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with goat anti-rat VR1 polyclonal IgG (1:1000, Santa Cruz Biotechnology). After being washed three times for 10 minutes with washing solution, the membranes were incubated with donkey anti-goat IgG-HRP (1:2000, Santa Cruz Biotechnology). The immunoreactive bands were visualized by an ECL Western blotting detection kit (Pierce Biotech) on light sensitive film.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was evaluated with SPSS software by one-way ANOVA followed by the Student-Newman-Keuls test for significance to compare the differences among various groups. Significance was accepted at $p<0.05$.

RESULTS

Effects of exogenous galanin on SP mRNA and SP release

The effects of galanin on SP mRNA and SP release in DRG cultures were investigated by RT-PCR and RIA, respectively. The ratio of SP mRNA/β-actin mRNA in galanin 1 nmol/L, 10 nmol/L, 100 nmol/L treated DRG neurons is 0.3497±0.0095, 0.3565±0.0107, 0.3562±0.0134, respectively. The ratio of SP mRNA/β-actin mRNA in control group is 0.3250±0.0127. There is no significance between each group. SP basal release in galanin 1 nmol/L, 10 nmol/L, 100 nmol/L treated DRG neurons and control group is 44.44±2.63 pg/ml, 46.14±2.78 pg/ml, 48.05±2.89 pg/ml, 42.98±1.94 pg/ml, respectively. Exogenous galanin sensitized capsaicin-evoked SP release, but did not have effects on SP mRNA and basal SP release (Figure 1, 2).

Effects of exogenous galanin on VR1 mRNA and VR1 protein expression

The effects of galanin on VR1 mRNA and VR1 protein expression in cultured DRG neurons were investigated by RT-PCR and Western blot, respectively. The ratio of VR1 mRNA/β-actin mRNA in galanin 1 nmol/L, 10 nmol/L, 100 nmol/L treated DRG neurons treated DRG neurons is 0.0868±0.0076, 0.1499±0.0110, 0.1800±0.0121, respectively. The ratio of VR1 mRNA/β-actin mRNA in control group is 0.0528±0.0066. The significance between each group indicated that VR1 mRNA expression increased in a dose-dependent manner of galanin in primary cultured DRG neurons. The relative density values of VR1 protein in galanin 1 nmol/L, 10 nmol/L, 100 nmol/L treated DRG neurons is 77.72±6.65, 97.02±4.96, 118.12±6.55, respectively. The relative density values of VR1 protein in control group is 56.99±4.81. Galanin promoted VR1 mRNA and VR1 protein expression in a dose-dependent manner in cultured DRG neurons after 4 days incubation (Figure 3, 4).

DISCUSSION

SP mRNA expression, basal SP release and capsaicin-evoked SP release, VR1 mRNA and VR1 protein expression influenced by exogenous galanin were studied in primary cultured embryonic rat DRG neurons. Although exogenous galanin could not induce SP mRNA and basal SP release, it could increase capsaicin-evoked SP release. And also,
exogenous galanin increased VR1 mRNA and VR1 protein expression in a dose-dependent manner. These results indicated that exogenous galanin promotes VR1 expression and then increases sensitivity of capsaicin on SP release from DRG neurons.

Galanin plays a role in the modulation of pain processing after nerve injury and peripheral inflammation [2, 8, 15, 17, 20]. SP is released upon appropriate stimulation and plays an important role in nociception and inflammatory actions [27, 30, 31]. Galanin immunoreactive cells are colocalized with SP immunoreactive cells in single sensory neurons of the rat DRG suggesting the functional significance of these neurotransmitters in the modulation of sensory action and neuropathic pain transmission [12, 32]. Both endogenous and exogenous appropriate stimulations are of great importance for neuropeptides expression or release of neurons in physiological or pathophysiological conditions [1, 24]. And also, different neuropeptides in single neurons may have functional interactions which are of importance for maintenance of proper function of the neurons [14]. Addition of exogenous galanin to the culture of DRG neurons did not affect SP mRNA and basal SP release in the present study, whereas it could increase capsaicin-evoked SP release which may be relevant to the increases of VR1 expression.

The non-selective cation channel VR1 is activated by pungent vanilloid compounds (such as capsaicin), extracellular protons or noxious heat. This ability to integrate thermal and chemical stimuli makes VR1 potentially well suited to modulate sensitivity of the nociceptor after tissue injury [5]. VR1 expression is regulated by many factors such as nerve growth factor and bradykinin [5, 24, 28]. In the present study, VR1 expression was modulated by administration of exogenous galanin at different concentrations. It might be that galanin binds to its own receptor GalR2 on sensory neurons. This event is consistent with the previous in vivo study that galanin-induced effects are mediated by GalR2 on capsaicin-sensitive primary sensory neurons and galanin can modulate VR1 function since GalR2 and VR1 are co-expressed in DRG neurons [10].

**Figure 1.** Effects of galanin on SP mRNA expression in primary cultured DRG neurons. SP and β-actin mRNA were analyzed by RT-PCR. Lane 1: Normal control. Lane 2: galanin 1 nmol/L. Lane 3: galanin 10 nmol/L. Lane 4: galanin 100 nmol/L. Bar graphs with error bars represent mean ± SEM (n=5).

**Figure 2.** Effects of galanin on SP release from DRG neurons induced by capsaicin. Galanin at different concentrations did not alter the basal SP release (p<0.05), but significantly enhanced SP release evoked by capsaicin. Bar graphs with error bars represent mean ± SEM (n=6).

'p<0.001 vs vehicle, †p<0.001 vs control, ‡p<0.05 vs galanin 1 nmol/L, Δp<0.001 vs galanin 1 nmol/L, †p<0.05 vs galanin 10 nmol/L.

* p<0.01 vs vehicle, # p<0.01 vs control, Δ p<0.05 vs galanin 1 nmol/L, ΔΔ p<0.01 vs galanin 1 nmol/L, § p<0.05 vs galanin 10 nmol/L.

![Figure 1](image1.png)

![Figure 2](image2.png)
Exogenous galanin at different concentrations from 1 nmol/L to 100 nmol/L did not affect SP mRNA expression as well as basal SP release during 4 days incubation. These results indicated that exogenous galanin could not initiate SP synthesis and release when cultured DRG neurons were at quiescent condition, whereas SP release was increased significantly when DRG neurons were stimulated by capsaicin after 4 days incubation with exogenous galanin at different concentrations as compared with that in control group. This result indicated that exogenous galanin could increase the capsaicin sensitivity on SP release. These in vitro results are in accordance with a biological role of galanin on capsaicin-evoked nociceptive behaviors.

The previous behavioral study of the peripheral function of galanin in capsaicin-induced inflammatory pain showed that ipsilateral intraplantar injection of galanin significantly increases capsaicin-evoked nociceptive behaviors in rats [10]. Both in vitro study in the present experiment and in vivo study of previous reports showed that galanin may be involved in nociception through acting on primary sensory neurons.

In conclusion, the in vitro data in the present study suggested that exogenous galanin may regulate capsaicin-evoked SP release from primary cultured DRG neurons. This finding could be relevant to the pro-nociceptive action of galanin in vivo. The effect caused by galanin might be that galanin modulates VR1 by binding to its own receptor GalR2 on sensory neurons and activating second messenger signaling cas-

**Figure 3.** Effects of galanin on VR1 mRNA expression in primary cultured DRG neurons. VR1 and β-actin mRNA were analyzed by RT-PCR. Lane 1: Normal control. Lane 2: galanin 1 nmol/L. Lane 3: galanin 10 nmol/L. Lane 4: galanin 100 nmol/L. Bar graphs with error bars represent mean ± SEM (n=5). *p<0.05 vs control, **p<0.001 vs control, †p<0.001 vs galanin 1 nmol/L, Δp<0.05 vs galanin 10 nmol/L.

**Figure 4.** Effects of galanin on VR1 protein expression in primary cultured DRG neurons. VR1 protein was analyzed by Western blot. Bar graphs with error bars represent mean ± SEM (n=5). *p<0.05 vs control, **p<0.001 vs control, †p<0.05 vs galanin 1 nmol/L, ††p<0.001 vs galanin 1 nmol/L, ‡p<0.05 vs galanin 10 nmol/L.
cades. The mechanism of galanin on capsaicin-evoked SP release is still to be clarified.

Disclosure statement

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