The Release of Substance P From Cultured Dorsal Root Ganglion Neurons Requires the Non-neuronal Cells Around These Neurons

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Abstract. Substance P is known to be released from dorsal root ganglion (DRG) neurons, but the possible involvement of non-neuronal cells in the process of substance P release is not known. During the culture period, the number of surviving DRG neurons, the total substance P content from the culture medium and DRG cells, and the total protein of DRG cells were monitored. Both the number of surviving neurons and the total substance P content decreased in a time-dependent manner, whereas the total protein synthesis was increased. The localization of substance P in small-to-medium–sized neurons was further confirmed by immunocytochemistry. Finally, the substance P release levels from the neuronal and non-neuronal enrichments were investigated by radioimmunoassay. Both partially purified DRG neurons and non-neuronal cells exhibited a weaker substance P release response to capsaicin or KCl, relative to unpurified DRG cells. The total substance P content from the partially purified DRG neurons was almost the same as that from the unpurified DRG cells, but much more than that from the partially purified DRG non-neuronal cells. These findings suggest that substance P is released from DRG neurons, and this process should require the coexistence of neurons and non-neuronal cells.

Keywords: capsaicin, dorsal root ganglion (DRG) neuron, non-neuronal cell, substance P content, substance P release

Introduction

Primary afferent neurons (also called sensory neurons) are highly specialized to convey sensory information from the periphery to the central nervous system. The cell bodies of the primary afferent neurons are found in the dorsal root ganglia, located in the intervertebral spaces. The peripheral processes of these neurons extend to the skin or muscles, and the central processes enter the spinal cord as the dorsal roots. Numerous studies have therefore focused on the signal transduction mechanisms of the dorsal root ganglion (DRG) neurons responsible for the modulation of pain transmission (1 – 3).

Substance P (a tachykinin peptide), a well-known neurotransmitter, is widely distributed in the central and peripheral nervous systems (4). It is released from primary afferent neurons to convey information about various noxious stimuli through a very complex process (5 – 8). Substance P is coded for by the preprotachykinin-A gene and is synthesized in the dorsal root ganglia (9). Substance P can play the important biological roles in neurogenic inflammation through binding to the specific G-protein–coupled neurokinin receptors, designated neurokinin-1, -2, and -3 receptors (10), as a primary afferent neuromodulator in nociceptive processes, namely, potentiating excitatory input to nociceptive neurons in the central and peripheral nervous systems (11 – 15). Several reports have shown that substance P is synthesized in the dorsal root ganglia (9, 16, 17), while no evidence excludes the existence of substance P in non-neuronal cells of the DRG. It is currently unknown whether the presence of non-neuronal cells is required for substance P release in response to various chemicals in cultured DRG cells.
fact, two-way communication between neurons and non-neuronal cells is essential for axonal conduction, synaptic transmission, and information processing in performing normal nervous system functions (18). Therefore, it is important to clarify the relationships between the substance P release, cultured DRG neurons, and non-neuronal cells.

In the present study, we used a double-labeling immunocytochemical technique to identify the localization of substance P in cultured DRG cells and used a highly sensitive radioimmunoassay to determine whether substance P release induced by applications of capsaicin and a high concentration of potassium chloride requires the coexistence of neurons and non-neuronal cells.

Materials and Methods

Materials

Dulbecco’s modified Eagle’s medium was from Nissui Pharmaceutical Co. (Tokyo). Horse serum, glutamine, and penicillin/streptomycin were from GibcoBRL (Gaithersburg, MD, USA). Trypsin (2.5%) was from Invitrogen Corporation (Burlington, ON, Canada). Mouse laminin was from Upstate Biotechnology (Lake Placid, NY, USA). Collagenase, polyethyleneimine and capsaicin, and potassium chloride were from Sigma Chemical Co. (St. Louis, MO, USA).

Isolation and culture of DRG cells

DRGs of adult Wistar rats (6 – 9 weeks of age) were dissociated into single isolated neurons and non-neuronal cells after being treated with enzymes (collagenase and trypsin) according to a previously described method (7, 8, 19). Next the cells (3 DRGs/dish) were plated on polyethyleneimine and laminin-coated 35-mm dishes and incubated in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated horse serum, 1% penicillin/streptomycin, and 200 mM glutamine. The cultures were maintained at 37°C in a water-saturated atmosphere with 5% CO₂ for 5 days before initiation of the experiments. On the fifth day of culture, neurons exhibited bright globular cell bodies containing either bi- or multi-polar neurites. Various non-neuronal cells such as Schwann cells, fibroblasts, and satellite cells were also present as an oxford gray cellular background. In these experiments, all procedures performed on animals were performed according to the Guide for Animal Experimentation, Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University, Japan.

Measurement of substance P content from the culture medium and in the cultured DRG cells

Except for some cultured cells treated with peptidase inhibitors alone (as a control), other cultured cells were exposed to capsaicin (100 nM) or to potassium chloride (50 mM) in 1 ml medium (serum-containing DMEM or Krebs-HEPES buffer) containing peptidase inhibitors (1 μM phosphoramidon, 4 μg/ml bacitracin and 1 μM captopril) for a designated period of time at 37°C in a water-saturated atmosphere with 5% CO₂. Thereafter, the substance P content collected from the culture medium and the cultured DRG neurons was measured by a highly sensitive radioimmunoassay (7, 20).

Survival ratio of the cultured DRG neurons

To examine the survival ratio of cultured DRG neurons, a field of view in each dish was randomly chosen under an Olympus IX71 inverted microscope with a 10X Ph1 objective (Olympus, Tokyo). In each chosen field of view, vital neurons at day 1 post-plating can be morphologically identified by their characteristic phase bright cell bodies containing either bi- or multi-polar neurites (21, 22), and the number of surviving neurons in the same field was again counted at days 2 – 5 post-plating. Finally, the survival ratio of the cultured DRG neurons in each stage was expressed as a percentage of the original number measured at day 1 post-plating.

Localization of substance P in cultured DRG cells

Immunocytochemical double-labeling was performed with anti-substance P and anti-MAP-2 (microtubule associated protein-2, a stringent marker for neurons) antibodies. Briefly, 4% paraformaldehyde-fixed cultured DRG cells on coverglasses were incubated with anti-substance P serum (1:1000 dilution; a gift of Dr. J.S. Hong, National Institute of Environmental Health Sciences, NIH, USA; ref. 23) for 1 h at room temperature, followed by treatment with Alexa Fluor 488-goat anti-rabbit IgG (1:1000 dilution; Molecular Probes, Eugene, OR, USA). After the substance P immunostaining, the coverglasses were boiled in 10 mM sodium citrate buffer (pH 6.0) for 5 min in a microwave oven (500 w) to unmask the MAP-2 antigen. After two 5-min washes in 0.01 M phosphate buffered saline, the coverglasses were incubated with an anti-MAP-2 primary antibody (1:200 dilution; Chemicon, Temecula, CA, USA) for 1 h at room temperature, followed by the incubation with Alexa Fluor 568 goat anti-mouse IgG (1:1000 dilution, Molecular Probes) for 1 h at room temperature. Finally, fluorescence signals were visualized with a microscope (IX71, Olympus), and images were captured by a CCD camera. According to the
Separation of neurons and non-neuronal cells from cultured rat DRG cells

The cultured DRG cells were enriched with the neuronal and non-neuronal cells by following the previously described method (24). Briefly, 15 h after the initial plating of DRG cells, the non-neuronal cells were firmly attached to the dish, while most of the neurons were only weakly adherent to the dish or to flattened non-neuronal cells. By carefully removing the culture medium from 6 dishes (35 mm), most dead cells and axonal and myelin debris were discarded prior to selectively dislodging the attached neurons with a gentle stream of culture medium (1.5 ml/dish) delivered from a pipette tip. After centrifugation of the cell suspension (4.5 ml) at 40 × g for 5 min, the cells were resuspended in serum-containing DMEM and then plated onto three new dishes (35 mm) for neuronal enrichment. For unpurified DRG cells, another aliquot from the same cell suspension (4.5 ml) containing neurons without centrifugation was equally replated on three dishes (1.5 ml/dish) in which the attached neurons were selectively dislodged. In comparison, the acquisition of non-neuronal enrichment was easy from three dishes in which the attached neurons were selectively dislodged. The cultures of unpurified DRG cells, partially purified neurons, and partially purified non-neuronal cells were continually maintained at 37°C in a water-saturated atmosphere with 5% CO₂ for 4 days before the initiation of the experiments.

Statistics

The data are presented as the mean ± S.E.M. Statistical analyses were performed by the multiple t-test with the Bonferroni correction following ANOVA. Significance was set at a value of P<0.05 (two-tailed).

Results

The relationship between the substance P content and the survival ratios of the cultured DRG neurons

The relationship between the substance P content in cultured DRG cells and the survival ratio of the cultured DRG neurons were investigated time-dependently. Under normal culture conditions, the uniformly dissociated DRG cells in 35-mm dishes were divided into two groups. Group I was used to demonstrate the survival ratios of the cultured DRG neurons, and group II was used to measure the substance P content and total protein. As shown in Fig. 1a, the amount of the total substance P content (including that in both the culture medium and the cultured cells) was also demonstrated to be a time-dependent decrease in the second group. Interestingly, the number of surviving DRG neurons was found to show a time-dependent decrease in the first group. In other words, the survival ratios of the cultured DRG neurons at each stage decreased in a time-dependent manner (Fig. 1b). Furthermore, using a modified Lowry protein assay (25), the amount of total protein from cultured DRG cells (including neurons and non-neuronal cells) in the same group of experiments was quantified to be a time-dependent increase (Fig. 1c). The source of total protein from each sample may therefore be considered to be neurons and non-neuronal cells. Based on these results, it was easy to determine that a very similar decrease pattern existed in both the amount of the total substance P content and the survival rate of these neurons and that the increase of the total protein was mainly caused by the proliferation of non-neuronal cells.

Localization of substance P in cultured DRG cells

To further illustrate whether only neurons expressed substance P, cultured DRG cells (including neurons and non-neuronal cells) were immunostained with the anti substance P serum. As shown in Fig. 2, double immunofluorescence staining thus indicated that the expression of substance P was detected in small-to-medium–sized neurons, but not in non-neuronal cells.

The release and total content of substance P from unpurified DRG cells, partially purified DRG neurons, and partially purified non-neuronal cells

Based on the procedure described in the Methods section, three cell populations were created including unpurified DRG cells, partially purified neurons, and partially purified non-neuronal cells, to investigate the involvement of non-neuronal cells in the process of substance P release from neurons (Fig. 3). The percentage of neurons in two randomly chosen fields per dish was calculated based on the total number of DRG cells counted in the same fields from three separate experiments. This demonstrated that the procedure for achieving neuronal and non-neuronal enrichments produced an approximately 5-fold decrease in total number of non-neuronal cells per 35 mm dish from the partially purified DRG neuron group (57 ± 4% of neurons per dish), compared with that from the unpurified DRG cell group.
Substance P Release From DRG Cells

Fig. 1. The relationship between the total substance P content and the survival ratio of cultured adult rat DRG neurons. a: Time-dependent decrease in the total substance P content from cultured DRG cells. b: Time-dependent decrease in the number of surviving neurons. c: Time-dependent increase in the total protein from cultured DRG cells. The data are expressed as the means ± S.E.M. (bars) from 3–4 (a), 9–10 (b), or 3 (c) separate experiments. *, **, and *** denote $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared with that on the first day after plating, respectively.

Fig. 2. Photomicrographs of substance P/MAP-2 double immunofluorescence in cultured adult rat DRG cells. a: Bright-field photomicrograph showing neurons and various non-neuronal cells in cultured adult rat DRG cells; b: photomicrograph showing MAP-2 immunoreactivity (red) in DRG neurons; c: photomicrograph showing substance P immunoreactivity (green) in DRG neurons; d: digitally merged photomicrographs from b and c. Bars: 50 µm.
It was simultaneously observed that there was an approximately 9-fold decrease in total number of neurons per dish from the partially purified DRG non-neuronal cell group (5 ± 1% of neurons per dish), compared with that from the unpurified DRG cell group. Interestingly, the total number of neurons from the unpurified DRG cell was almost the same as that from the partially purified neuron group. These three specific cell groups were used to examine possible changes in the release and total content of substance P after a short-term stimulation with capsaicin or KCl. Initially, the amount of substance P release from the unpurified DRG cells, the partially purified DRG neurons, and the partially purified non-neuronal cells was examined with or without 100 nM capsaicin or 50 mM KCl for 10 min (Fig. 4a). The results showed that both capsaicin and KCl evoked a significant release of substance P from the unpurified DRG cells (82.9 ± 17.9 and 124.6 ± 24.9 pg/dish, respectively), whereas these potent stimulators of substance P release did not easily evoke substance P release from either the partially purified neurons (17.9 ± 7.0 and 41.2 ± 10.2 pg/dish, respectively) or the partially purified non-neuronal cells (18.9 ± 5.8 and 19.8 ± 2.3 pg/dish, respectively). Interestingly, the amount of total substance P content from the unpurified DRG cells was almost the same as that from the partially purified neurons at the end of the substance P release experiments (Fig. 4b). However, the amount of total substance P content from the partially purified DRG non-neuronal cells was about 10% of that from the unpurified DRG cells.

**Discussion**

The present study provided important evidence including the exact localization of substance P and time-dependent changes in the total substance P content, the survival ratios of the cultured DRG neurons, and total protein. In addition, it demonstrated that the non-neuronal cells are essential for the substance P release from the cultured DRG neurons, even though the non-neuronal cells do not contain substance P.

Previous studies (8, 26, 27) described “substance P release from DRG neurons”, but did not provide direct and concrete evidence to explain why substance P is released from neurons, and not from non-neuronal cells. Therefore, to explain the reason for substance P
being released from DRG neurons, these experiments primarily analyzed the correlation between the substance P content in cultured DRG cells and the survival ratios of the cultured DRG neurons. During the culture of DRG cells (Fig. 1), significant reductions in the substance P content and the survival ratios of the DRG neurons were observed. The relationship between the amount of total protein and substance P content in cultured DRG cells was investigated during the culture of DRG cells. Protein is the basic structural material of all cells, so the amount of total protein in the cells per dish is therefore useful as an indirect index to reflect the total cell number per dish for each experiment. Moreover an increase in the amount of total protein is caused by the proliferation of non-neuronal cells because the adult neurons did not proliferate under the experimental conditions used (Fig. 1). This indicated that there was a significant increase in the total number of cultured DRG non-neuronal cells during the culture of DRG cells. These data suggest that the substance P may be present in DRG neurons only. To further illustrate the accuracy of this observation, it was necessary to examine whether the substance P content is only expressed in DRG neurons by immunochemistry. This demonstrated the expression of substance P in DRG neurons, not in non-neuronal cells (Fig. 2), which are responsible for the regulation of substance P release. Interestingly, the expression of substance P was mainly observed in the cytoplasm of small-diameter DRG neurons, based on the criteria for large (a diameter >40 µm) and small (a diameter <25 µm) DRG neurons (28). Therefore, substance P is probably produced in the cultured small-to-medium-sized DRG neurons, thus suggesting that substance P is released from cultured DRG neurons, not from DRG non-neuronal cells.

Non-neuronal cells (glial cells including Schwann and satellite cells and others), originally thought to provide support and protection for neurons, are now recognized as an important modulator of pain signaling (29–31) in the nervous system. Under these experimental conditions, the cultured DRG cells include neurons, Schwann and satellite cells, and fibroblasts (Fig. 3). This suggests that the cultured DRG non-neuronal cells might be involved in the process of substance P release from cultured DRG neurons. Therefore, in the present study, we used two potent stimulators of substance P release. One is a high concentration of KCl (potassium chloride) because it is a much more effective agent for depolarizing the membrane and it is often used to evoke substance P release (8). Another is a high concentration of capsaicin because it can directly activate transient receptor potential vanilloid receptor subtype 1 (TRPV1) to cause the release of substance P from cultured DRG neurons (6, 8). As shown in Fig. 4a, 50 mM KCl produced a more intense substance P release response than 100 nM capsaicin in the unpurified DRG cells. However, both 50 mM KCl and 100 nM capsaicin induced a lower level (approximately 3-fold weaker) of substance P release from the partially purified DRG neurons than the unpurified DRG cells (Fig. 4a). In view of the results shown in Fig. 4a and the above-described observations, the difference in the substance P release induced by 50 mM KCl and by 100 nM capsaicin from the partially purified neurons may be explained as a consequence of their pharmacological actions under our experimental conditions. This difference might be further expected to gradually disappear following a decrease in the number of non-neuronal cells in the partially purified DRG neurons group. Moreover, the
total substance P content from the partially purified DRG neurons was almost the same as that from the unpurified DRG cells, but much more than that from the partially purified DRG non-neuronal cells (Fig. 4b). These data suggest that the decrease in DRG non-neuronal cells results in an inhibitory effect on the substance P release from cultured DRG neurons. In other words, the process of the substance P release from cultured DRG neurons requires the physiological ratio of neurons and non-neuronal cells under these experimental conditions.

Recently, the important roles of Schwann cells have been elucidated in multiple processes including synapse formation, function and maintenance, and repair (32), and the release of various endogenous substances such as chemoattractants, proinflammatory cytokines, reactive oxygen species, and nitric oxide (33). For example, Schwann cells can detect the evoked synaptic activities and modulate the synaptic transmission by elevating the intracellular Ca\(^{2+}\) levels (34, 35). Furthermore, it is already well known that Ca\(^{2+}\) entry through Ca\(^{2+}\) channels including (voltage-gated Ca\(^{2+}\) channels, TRPV1, and others) plays an important role in modulating the release of transmitters (36, 37) including substance P (38). Therefore, Schwann cells are thus considered to be important in the regulation of sensory neuron function. However, in the process of the substance P release from cultured DRG neurons, the possible role(s) of non-neuronal cells (including Schwann and satellite cells), for example, contributing to the process of chemical communication (18, 39), still must be elucidated by further studies.

In conclusion, this study demonstrated the existence of substance P in cultured DRG neurons, not in non-neuronal cells, and clarified the involvement of DRG non-neuronal cells in the process of substance P release from cultured DRG neurons. These observations provide important evidence to help us better understand the necessity and importance of non-neuronal cells in the process of substance P release from primary afferent neurons.

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