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Expression and release of progalanin in fibroblasts

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ABSTRACT

Galanin is a neuropeptide expressed in the central and peripheral nervous systems. Galanin is known to be biosynthesized in neural and endocrine cells, but little evidence exists for its synthesis in other cells. In this study, we explored galanin-releasing nonneural cells using radioimmunoassay, finding that some fibroblasts produced and released the galanin-like immunoreactive component (galanin-LI). The molecular weight of the galanin-LI obtained from the fibroblasts, as measured by gel filtration chromatography and Western blotting, was 14 kDa and suggested that the compound was progalanin. Peptide mass fingerprinting analysis identified the large form of galanin-LI as progalanin without its signal sequence. In addition, galanin-LI was located in the Golgi bodies and vesicle-like structures of the fibroblasts. Furthermore, the addition of brefeldin A, an inhibitor of transport from the ER, decreased the release of galanin-LI. In this study, we showed that the fibroblast, a nonneural and nonendocrine cell type, produced and released a galanin precursor, progalanin, without processing via Golgi bodies or secretory vesicles.

1. Introduction

Galanin is a neuropeptide of 29 or 30 amino acid residues that was first isolated from porcine intestine [1]. Galanin has now been cloned from several species [2–15]. The sequence of preprogalanin contains a signal sequence, progalanin-message peptide (PGMP), galanin, and a galanin message-associated peptide (GMAP). Porcine, bovine, canine, and rodent galanin sequences all possess a C-terminal amide and consist of 29 amino acids, but human galanin lacks a C-terminal amide and consists of 30 amino acids. The N-terminal sequence of galanin from positions 1 to 15 is common among previously reported mammalian sequences.

Galanin is widely distributed in the central and peripheral nervous systems. The peptide is located in the hypothalamus, brain stem, and spinal cord, primarily in nerve terminals [16–23]. In many systems, galanin acts as an inhibitory hyperpolarizing neuromodulator by binding specific G-protein-coupled receptors (GPCRs). Three galanin receptors, GAL_1 [24], GAL_2 [25], and GAL_3 [26], are expressed in the central and peripheral nerve systems. In the peripheral tissue, galanin is involved in axotomy [27–29] and chronic nerve compression [30,31], and peripheral nerve injury [32] induces increased galanin expression in dorsal root ganglion neurons.

In the peripheral tissue, some nonneural cells also express a galanin-like immunoreactive component (galanin-LI), including the nerve terminals and fibers of the dermis [33], keratinocytes [34,35], follicular and interfollicular epidermal cells, ductal cells of the eccrine sweat glands [33,34], and fibroblast-like cells during granulation [36]. However, the molecular weight of the galanin-LI in nonneural cells was 15.4 kDa, much greater than that of galanin (3 kDa). The aim of this study was to explore those cells releasing galanin-LI and to examine the molecular form of galanin in nonneural cells.

2. Materials and methods

2.1. Materials

Lysozyme, chloramine-T, brefeldin A (BFA), and $DiOC_6(3)$ were purchased from Sigma Chemical Co., USA. Eagle's minimum essential medium (MEM) was a product of the Nissui Pharmaceutical Co., Ltd., Japan. MCDB-104 was purchased from Nihon Pharmaceutical Co., Ltd., Japan. Fetal bovine serum (FBS) was purchased from Moregate, Australia & New Zealand. Normal rabbit serum and goat anti-rabbit γ-globulin serum were products of the Daiichi Radioisotope Lab., Chiba, Japan. Rhodamine-conjugated anti-mouse antibody was purchased from BioSource, Camarillo, CA. Normal goat serum was purchased from Vector Lab., Ltd., USA. [^{125}I]-iodine was purchased from Amersham Pharmacia Biotech, UK. Human galanin was purchased from Peptide Institute, Inc., Osaka, Japan.

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2.2. Cells and cell culture

The following normal cell lines were used in the present study: human fibroblasts (ASF-4-1, TIG-1 [37], TIG-3 [38], TIG-7 [39], KMS-6 [40], MRC-5 [41], IMR-90 [42], KT4-T1, and WI-38 [43]) and immortalized cells (KMST-6 [40] and WI-38VA13), mouse fibroblasts (Balb/c 3T3), Chinese hamster fibroblasts (V-79), chick fibroblasts (KHT-1, -2, -4, and -6, established by K.K.), human keratinocytes (NHEK(B), breast, product No. KK-4101, Kurabo Industries, Tokyo, Japan), and human umbilical vein endothelial cells (HUVEC, KT4-1, established by K.K.). The normal cell lines were used within population doubling levels (PDL) of 23–40. The cells were cultured in MEM supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cell numbers were counted with a Model Z1 Coulter Counter (Coulter Electronics, Inc. Hialeah, FL).

2.3. Radioimmunoassay

The radioimmunoassay (RIA) was performed at 4 °C as described previously [44]. R0672 and R0901 antibodies were raised in rabbits against synthetic human galanin peptides 1–15 (N-terminal region) and 9–30 (C-terminal region), respectively.

2.4. Cell extract and culture medium

The cell extracts were prepared as follows. KMS-6 and WI-38 cells were homogenized in a fivefold volume of 0.1 M acetic acid using a Teflon glass homogenizer and then boiled for 10 min. After the addition of 1 M acetic acid, the homogenate was centrifuged at 3000 rpm for 30 min at 4 °C. The culture medium was also centrifuged at 3000 rpm for 30 min at 4 °C. The supernatants of the cell extracts were lyophilized and stored at −20 °C until RIA.

2.5. RT-PCR analysis

Total RNA was prepared from the KMS-6 and WI-38 cells using an SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA). Genomic DNA was removed with DNaseI. cDNA was synthesized using Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions. Specific primers were designed to amplify human galanin (5′-GACCCCGACGCT CCGAACC-3′ and 5′-GCTCTCAGGACCGCTCGATGT-3′), and human β-actin as a positive control. The PCR protocol was as follows: initial denaturation at 95 °C for 5 min, followed by 30 to 45 cycles (depending on primer) of 94 °C for 1 min, 58 °C–62 °C for 1 min, and 72 °C for 1 min. PCR products were electrophoresed on ethidium bromide—containing 1.2% agarose gel and visualized under UV illumination. The primers for β-actin were also used to assess the integrity of RNA preparation.

2.6. Immunocytochemistry

KMS-6 cells were plated on culture slides (FALCON, USA). After incubation for 48 h, the cells were fixed with 4% paraformaldehyde for 10 min. After being blocked with normal goat serum (×50) for 30 min at room temperature, the cells were incubated with monoclonal antibody B4G9, raised against galanin (1–15) (established in our laboratory, 1 μg/ml, specificity shown in previous report [36]) overnight at 4 °C. The anti-galanin(1–15) monoclonal antibody B4G9 was preincubated with 5 μg/100 μl of galanin for 1 h at room temperature and used as an adsorption control and then with a secondary rhodamine-conjugated anti-mouse goat antibody (×250, BioSource International, Camarillo, CA) for 30 min at room temperature. The cells were then stained for the endoplasmic reticulum (ER) with 2.5 μg/ml $DiOC_6(3)$ for 30 s at room temperature [45]. Fluorescence was viewed with a laser scanning confocal microscope (LSM510, Carl Zeiss Co. Ltd., Oberkochen, Germany). Hoechst 33342 nucleic acid stain (Sigma-Aldrich, St. Louis, MO) was used to observe the nucleus.

2.7. Gel filtration

Gel filtration was conducted on a Sephadex G-50 fine column (1.0 × 100 cm, Pharmacia, Uppsala, Sweden) using 1 M acetic acid as an eluent. Each fraction of 0.9 ml was collected and lyophilized. The lyophilized fractions were dissolved with the standard diluent for RIA. The column was calibrated with BSA (molecular weight 69 kDa, Kav 0), lysozyme (14.4 kDa), insulin (6 kDa), human galanin (3 kDa) and dbcAMP (Kav 1.0).

2.8. Inhibition of the classical ER/Golgi secretion pathway

To investigate the secretory pathway of galanin precursor in fibroblasts, brefeldin A (BFA) was used. After confluence, KMS-6 cells were treated with 1 μg/ml BFA. After 24 h of incubation, the medium was removed and lyophilized. The cells were cultured in a new medium containing BFA for another 24 h, and the medium was then collected and lyophilized. As a control, cells were cultured without BFA in the same manner. As BFA competes with RIA for galanin-LI, BFA was removed from the cultured medium by centrifugal gel filtration prior to RIA. In brief, the lyophilized medium was dissolved in 1 M acetic acid (500 μl) and loaded on a Sephadex G-10 gel suspension (8 ml) packed in a 10 ml plastic syringe. A BFA-free run-through fraction was collected by centrifugation at 800 rpm and 4 °C for 5 min and lyophilized for use in RIA. The remaining cells were used for immunocytochemistry and RT-PCR.

2.9. Partial purification of the galanin-like substance

Galanin-LI was partially purified by ultrafiltration and Cibacron Blue-liganded affinity chromatography. Lyophilized medium (100 ml, containing approximately 6 pmol of galanin-LI) was dissolved in 1 M acetic acid and centrifuged at 7500 rpm for 30 min. The supernatant was centrifuged in a Vivaspin cartridge (molecular weight cut 30 kDa, Vivascience, Hannover, Germany). The filtrate was loaded on a Sep-PacC18 cartridge (Waters, Milford, MA), eluted with 1 M acetic acid, and dialyzed against 50 mM potassium phosphate (pH 7.0) in Spectra/Por dialysis tubing (molecular weight cut 3.5 kDa, Spectrum Laboratories, Rancho Dominguez, CA). The dialyzed sample was carried on a HiTrap Blue HP column (1 ml, GE Healthcare UK Ltd, England). The fraction eluted with 50 mM potassium phosphate (pH 7.0) was dialyzed against 100 mM ammonium bicarbonate, lyophilized, and stored at −80 °C.

2.10. Polyacrylamide gel electrophoresis

2.10.1. One-dimensional electrophoresis

One-dimensional SDS-PAGE was performed according to the methods of Laemmli [46] using a Mini-Protean 3 Cell electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA). Galanin-LI, partially purified from 50 ml of cultured medium, was subjected to electrophoresis (15% (w/v) polyacrylamide gel).

2.10.2. Two-dimensional electrophoresis

A galanin-LI preparation partially purified from 100 ml of culture medium was loaded in a gel tube (3 mm diameter, 85 mm length) with a linear pH 4–10 Immobiline (GE Healthcare UK Ltd., England) gradient [47]. The gel tube was run overnight at 4 °C using increasing voltage (200–2000 V) in an NA-1616 isoelectrofocusing system (Nihon-Eido, Tokyo, Japan). The forced gel was equilibrated with Laemmli's running buffer and laid on the surface of a 1 mm thick 15% (w/v) polyacrylamide gel.