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The secretory response through electric stimulation of differentiated PC12 rat pheochromocytoma cells transfected with neuropeptide Y fused with enhanced green fluorescent protein

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Abstract

Exocytosis in pheochromocytoma cells was induced by electric stimulation. To chase the movement of vesicles by electric stimulation, dense-core secretory vesicles were visualized by expression of the fusion protein between neuropeptide Y and enhanced green fluorescent protein (EGFP) in these differentiated PC12 rat pheochromocytoma cells. When the cells were stimulated with constant voltage potential at −300 mV, the movement of dense-core secretory vesicles could be regulated.

Introduction

Neurotransmitters are released into the synaptic cleft when there is fusion of synaptic vesicles with the plasma membrane, similar to the mechanism where various physiologically active substances, including hormones and cytokines, are secreted from cells. This secretory process occurs at the synaptic endings and involves targeting, docking, priming, fusion, and endocytosis. Synaptic vesicles containing neurotransmitters move to the active zone (targeting), join with the presynaptic membrane near a Ca^{2+} channel (docking), and then grow and mature (priming) (Holz *et al*. 1989, Heuser & Reese 1973). Priming vesicles then fuse with the plasma membrane and release neurotransmitters into the synaptic cleft (exocytosis) when Ca^{2+} uptake is induced (Heinemann *et al*. 1994).

Presynaptic terminals contain dense-core secretory granules which are necessary for the secretion of hormones and neuropeptides. Dense-core secretory granules are also found in neuroendocrine cells. PC12 rat pheochromocytoma cells are good models for the study of neurosecretion; in these cells exocytosis of dense-core granule uses the same machinery which functions in synaptic neurotransmission (Martin 1994).

We have found that electric stimulation can regulate various cellular functions, such as differentiation, proliferation, and gene expression (Koyama *et al*. 1996, 1997, Kimura *et al*. 1998a,b). Electric stimulation causes various cellular responses by inducing Ca^{2+} influx (Kimura *et al*. 1998a,b). Therefore, exocytosis of dense-core granules, which is caused by inducing Ca^{2+} influx, can be regulated by electric stimulation.

To estimate the effect of electric stimulation on cellular secretion, it is necessary to monitor both granule movement and exocytosis. For this purpose we used enhanced green fluorescent protein (EGFP), which has been proven to be a useful tool for visualizing Ca^{2+} triggered secretion (Pouli *et al*. 1998, Imaizumi *et al*. 2002). Lang *et al*. (1997) visualized dense-core granules of PC12 cells by recombinant EGFP fusion with the neurotransmitter, neuropeptide Y (NPY). Expression of the fusion protein caused bright fluorescence

in dense-core granules allowing the secretions to be imaged.

In this study we have attempted to observe the movement of dense-core secretory granules and exocytosis induced by electric stimulation, by using the fusion protein between NPY and EGFP (NPY-EGFP).

Methods

Plasmid construction

The plasmid, pNPY-EGFP, was constructed as an expression vector to localize the fusion protein, NPY-EGFP, in synaptic vesicles in PC12 cells. A DNA fragment coding enhanced green fluorescent protein (EGFP) was obtained from pEGFP-1 digested with *EcoR*I and *Not*I. This fragment was subcloned into pcDNA3.1 (pcDNA-EGFP). A structural gene of neuropeptide Y (NPY) was amplified by PCR using the forward primer 5′-CCC AAG CTT ATG ATG CTA GGT AAC AAA CG-3′, including a *Hind*III site, and reverse primer 5′-CAG AAT TCT CCA CAT GGA AGG GTC TTC AA-3′, including an *EcoR*I site. The PCR product was digested and subcloned into the plasmid pcDNA-EGFP (pNPY-EGFP).

Cell culture and transfection

PC12 cells were cultured in RPMI 1640 medium with 5% (v/v) fetal bovine serum and 10% (v/v) horse serum on type IV collagen-coated dish. PC12 cells were transfected using a standard cationic lipid-based DNA delivery protocol. NPY-EGFP expression cells were selected by antibiotic G418. Synaptic vesicles were contained in differentiated PC12 cells but not in the non-differentiated PC12 cells. Therefore, differentiation of PC12 cells with neurites was induced by exchanging low serum medium containing 30 ng nerve growth factor ml^{-1}.

ELISA

PC12 cells were grown in 6-well multiplates (1×10^5 cells $well^{-1}$) and incubated in serum-free medium. These cells were treated with either 100 mM KCl or the same amount of serum-free medium, and incubated for 15 min. The medium was collected and assayed by ELISA. Anti-EGFP antibody (Santa Cruz Biotechnology, Inc.) and alkaline phosphatase-labeled anti-rabbit IgG antibody (COSMO BIO) were used as the primary and secondary antibodies, respectively.

Electric stimulation

The electric stimulator consists of three electrodes, a reference electrode (Ag/AgCl), a counter electrode (Pt), and a working electrode (indium tin oxide, ITO). Cells were cultured on the working electrode and the electrical potential was applied with a potentiostat.

Measurement of EGFP fluorescence

The fluorescence image of the emitted light was visualized with a 100× oil immersion objective, and detected on an optical unit, the ARGUS-50 systems (Hamamatsu Photonics, Hamamatsu, Japan), where the camera output was also digitized and analyzed. The images of EGFP expressed cells were collected at every min and individual cells were illuminated for 1 s.

Results and discussion

Expression of the fusion protein between NPY and EGFP in PC12 cells

PC12 cells were transfected with plasmids, pcDNA-EGFP and pNPY-EGFP, by lipofection and differentiated into neuron-like cells in the presence of nerve growth factor. PC12 cells transfected with pcDNA-EGFP showed fluorescence throughout the cells (Figure 1A). Whereas PC12 cells transfected with pNPY-EGFP showed punctate fluorescence in the cytosol and neurite, but not in the nucleus (Figure 1B). In addition, Lang *et al.* (1997) confirmed by immunohistochemistry that a fusion protein of chromogranin B (which is dense-core granules matrix protein) with GFP served to localize dense-core granules in PC12 cells by tagging these specific protein granules and observing EGFP fluorescence. This directly reflected the dynamics of these granules. Therefore, by using this method PC12 cells labeled with NPY-EGFP can be a useful tool in monitoring the dynamics of these granules on various types of stimulation.

Confirmation of secreted EGFP

To confirm the release of localized NPY-EGFP from dense-core vesicles the amount of NPY-EGFP released from cells was determined by ELISA. When PC12 cells transfected with pNPY-EGFP were stimulated by 100 mM KCl, the amount of NPY-EGFP released in the medium was approx. 5-fold higher than

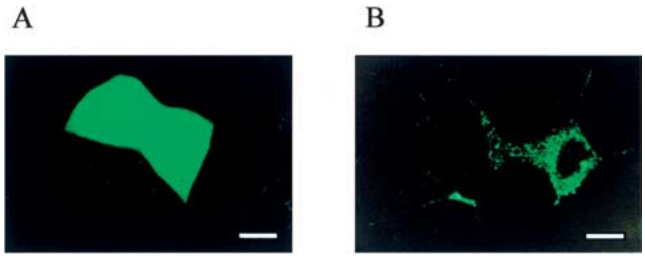

Fig. 1. Localization of NPY-EGFP in differentiated PC12 cells. NPY-EGFP localized in dense-core secretory vesicles. (A) Single PC12 cells were transfected with pcDNA-EGFP. Plasmid pcDNA-EGFP constitutively expressed EGFP in PC12 cells. EGFP was found throughout the cells. (B) Single PC12 cells transfected with pNPY-EGFP, in which NPY functioned to localize EGFP to dense-core secretory vesicles, showed punctate fluorescence in the cytosol and neurite (scale bar: 10 μm).

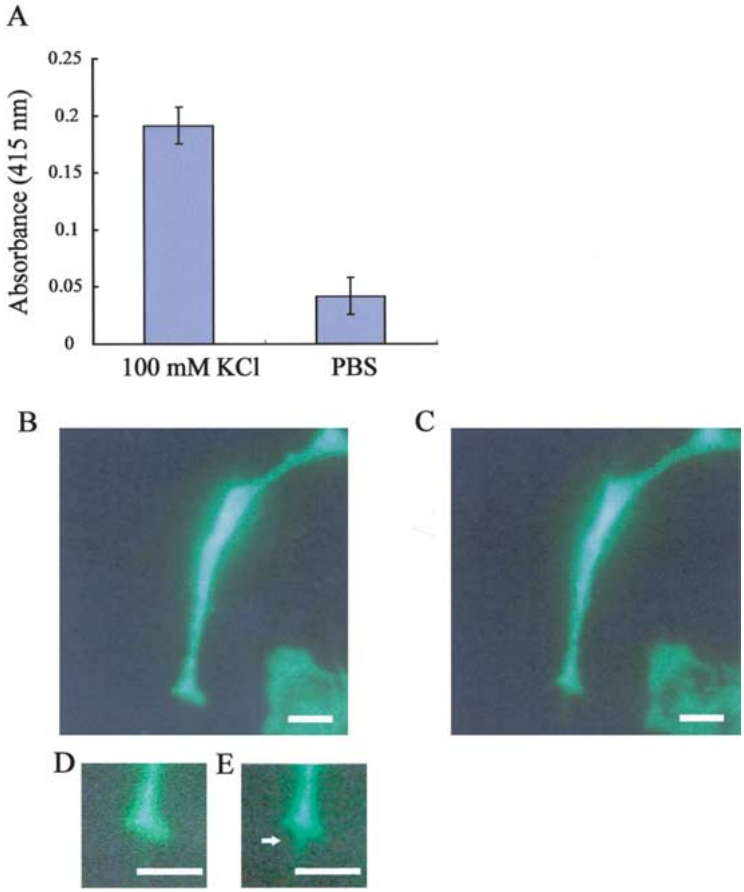

Fig. 2. Secretion of NPY-EGFP from the neurites of PC12 cells. Release of NPY-EGFP localized in dense-core secretory vesicles are confirmed by ELISA and observed. (A) NPY-EGFP release from PC12 cells transfected pNPY-EGFP was measured by ELISA. The result was shown at an absorbance value at 415 nm. (B) Neurite localized EGFP was stimulated by 100 mM KCl (C). Shown are the magnified images in the top of the neurite before (D) and after (E) 100 mM KCl stimulation. Arrow indicates the leak of EGFP fluorescence (scale bar: 10 μm).

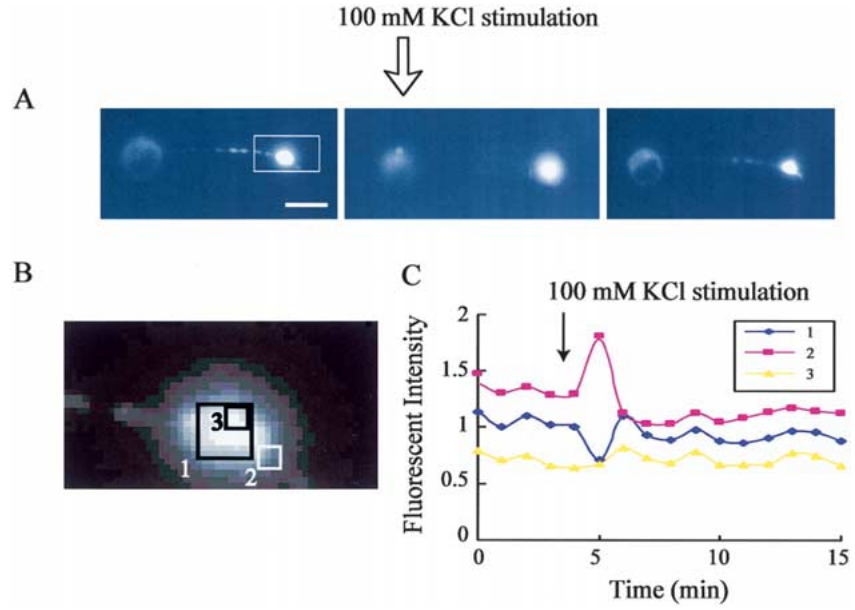

Fig. 3. Fluorescent change derived from vesicle movement caused by stimulation with 100 mm KCl. The localization of dense-core secretory vesicles visualized by NPY-EGFP changed after stimulation with 100 mm KCl. EGFP fluorescent images are shown using the ARGUS50 system (A) (left panel: before stimulation, center panel: on stimulation, right panel: after stimulation). (B) The magnified image in (A), surrounded with the white box, shows the top of the neurite. Box 1 shows the entire neurite, while box 2 shows the edge of neurite and box 3 shows a part of neurite. (C) Here the fluorescence changes of each box of (B) are shown and were calculated using the ARGUS50 system. The EGFP fluorescence change was measured at every min. Relative activity was shown in reference with the initial value. Arrows indicate the 100 mM KCl injection (scale bar: 10 μm).

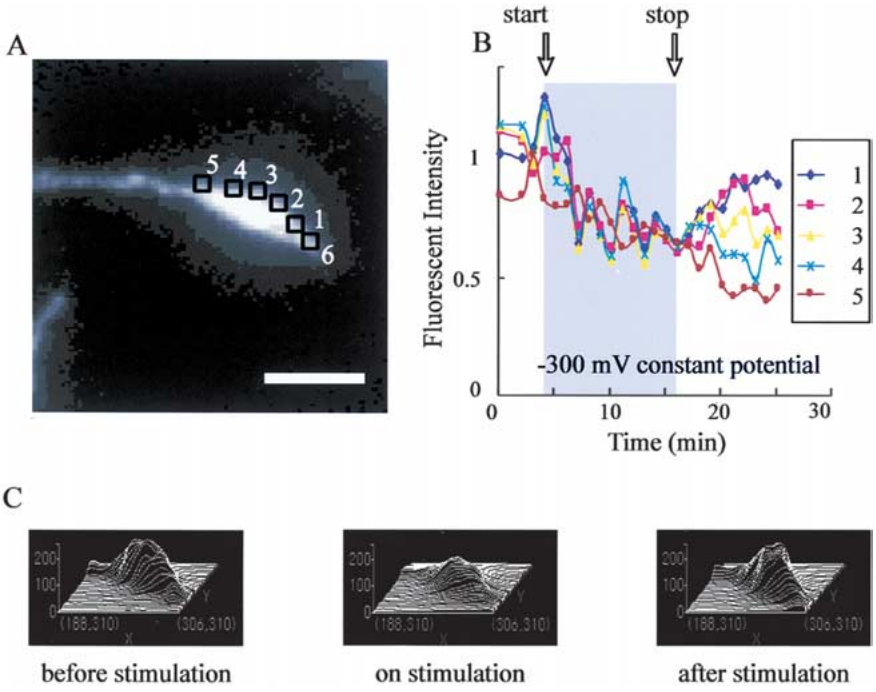

Fig. 4. Fluorescent change by electric stimulation. The localization of dense-core secretory vesicles visualized by NPY-EGFP was changed by stimulation with electric stimulation. (A) The point of measurement at the tip of neurite is shown in each box. (B) Fluorescence change induced by electric stimulation in each box was calculated by the ARGUS50 system. Relative activity was shown in reference with the initial value. The gray zone indicates an electric stimulation. (C) The three-dimensional images of the intensity of EGFP fluorescence at the tip of a neurite. EGFP fluorescence change was measured every 2 min. Relative activity was shown in reference with the initial value (scale bar: 10 μm).

that of the non-stimulated cells (Figure 2A). Figure 2B shows the fluorescent image at the neurite of the differentiated PC12 cells. When PC12 cells transfected with pNPY-EGFP were stimulated by 100 mM KCl, we could observe the decay of EGFP fluorescence in the entire neurite (Figure 2C) and the secretion event at the tip of the neurite (Figure 2E). This picture was taken on long exposure time (approx. 5 s), such that the fluorescence accumulation enabled observation of the exocytosis event.

Movement of synaptic vesicles by KCl and electric stimulation

To measure the change of EGFP fluorescence over a time course, a series of successive images was taken using the ARGUS50 system (Hamamatsu Photonics). Figure 3A shows the fluorescent images of NPY-EGFP in PC12 cells before and after stimulation with 100 mM KCl. Before stimulation (left panel) dense-core granules at the top of the neurite were closely localized. However, soon after stimulation (center panel) the fluorescence was extensively diffused to the edge of the neurite, and was re-localized 15 min after stimulation (right panel). Next, we measured the change in EGFP fluorescence in the top of the neurite at every minute, which corresponds to the boxed image in Figure 3B. EGFP fluorescence in the entire neurite (Figure 3B, box 1) decreased immediately, and soon after reached a constant value (Figure 3C). The EGFP fluorescence in box 2 (Figure 3B), which indicates the tip of the neurite, increased initially and then decreased. The EGFP fluorescence in box 3 (Figure 3B) did not change (Figure 3C). As shown in Figure 2, depolarization signals induced by KCl facilitated EGFP secretion to the exterior of cells. This phenomenon is shown in Figure 3B, box 2. The fluorescent changes, synchronized with each box at 2 min and 9 min, are background signals. These results indicate that the EGFP fluorescence released from the tip of the neurite was measured as the fluorescence intensity increased. The measurement of EGFP fluorescence intensity in the transfected PC12 cells thus makes it possible to monitor both vesicle movement and exocytosis.

We measured then the change of EGFP fluorescent intensity by stimulating with a -300 mV constant potential, corresponding to the boxed images in Figure 4A, which pertains to the edge of the neurite. The intensity of EGFP fluorescence decreased, reaching a constant value soon after electric stimulation (gray zone in Figure 4B). Figure 4C shows three-dimensional images of the EGFP fluorescence intensity of the neurite from Figure 4A. When transfected PC12 cells were stimulated by -300 mV constant voltage, dense-core granules with EGFP fluorescence showed a homogenous distribution (Figure 4C, center panel). However, the cessation of electric stimulation induced them to re-localize (Figure 4C, right panel). These results find that electrical stimulation could regulate the movement of dense-core granules at the neurite of PC12 cells. Furthermore physical mobilization of dense-core secretory vesicles facilitates neuropeptide release from differentiated PC12 cells (Yuen-Keng *et al.* 2002). Therefore it is suggested that electric stimulation may control neuropeptide release through regulating the movement of dense-core secretory vesicles.

This research describes a method to regulate movement of dense-core granules in differentiated PC12 cells transfected with NPY-EGFP by constant potential electric stimulation. This method may serve as a useful tool to regulate the secretion of physiologically active substances to the cell exterior.

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