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The small GTPase Cdc42 modulates the number of exocytosis-competent dense-core vesicles in PC12 cells

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ABSTRACT

Although the small GTPase Rho family Cdc42 has been shown to facilitate exocytosis through increasing the amount of hormones released, the precise mechanisms regulating the quantity of hormones released on exocytosis are not well understood. Here we show by live cell imaging analysis under TIRF microscope and immunocytochemical analysis under confocal microscope that Cdc42 modulated the number of fusion events and the number of dense-core vesicles produced in the cells. Overexpression of a wild-type or constitutively-active form of Cdc42 strongly facilitated high-KCl-induced exocytosis from the newly recruited plasma membrane vesicles in PC12 cells. By contrast, a dominant-negative form of Cdc42 inhibited exocytosis from both the newly recruited and previously docked plasma membrane vesicles. The number of intracellular dense-core vesicles was increased by the overexpression of both a wild-type and constitutively-active form of Cdc42. Consistently, activation of Cdc42 by overexpression of Tuba, a Golgi-associated guanine nucleotide exchange factor for Cdc42 increased the number of intracellular dense-core vesicles, whereas inhibition of Cdc42 by overexpression of the Cdc42/Rac interactive binding domain of neuronal Wiskott–Aldrich syndrome protein decreased the number of them. These findings suggest that Cdc42 facilitates exocytosis by modulating both the number of exocytosis-competent dense-core vesicles and the production of dense-core vesicles in PC12 cells.

1. Introduction

Peptide hormones are packed into dense-core vesicles at the Golgi complex and are released to the extracellular space in response to Ca^{2+} influx. After the budding from the Golgi complex, the dense-core vesicles translocate to the plasma membrane (transport step). The transported dense-core vesicles morphologically attach to the plasma membrane (docking/tethering step). The vesicles prepare to fuse with the plasma membrane with tight complexes containing soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein (priming step), and the readily releasable dense-core vesicles fuse with the plasma membrane following Ca^{2+} influx (fusion step) [1]. To maintain a continuous exocytosis, the refilling of the readily releasable pool by biogenesis and mobilizing the dense-core vesicle pools occurred constantly.

Refilling of the readily releasable pool has been shown to be regulated by trafficking of the dense-core vesicles to the plasma membrane, and this has been implicated in coordinated re-organization of actin filaments [2–4]. Although small GTPase Rho family proteins are involved in actin remodeling, the intracellular distribution and their function in exocytosis are significantly distinct. For example, Cdc42 and Rac1 preferentially exist in the subplasmalemmal region and facilitate exocytosis, whereas RhoA is present on the dense-core vesicles and inhibits exocytosis [5]. In addition to the actin reorganization by Rho family proteins, in particular, Cdc42 has been shown to bind to SNARE proteins directly or indirectly in insulin-secreting cells [6,7]. Therefore, we focused on the role of Cdc42 on the dense-core vesicle exocytosis in neuroendocrine PC12 cells.

In the present study, to clarify the role of Cdc42 on the dense-core vesicle exocytosis, we used total internal reflection fluorescence (TIRF) microscopy to analyze single exocytotic events at a high spatiotemporal resolution. We found that overexpression of a

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wild-type or constitutively-active form of Cdc42 strongly facilitated high-KCl-induced exocytosis from the newly recruited plasma membrane vesicles in the PC12 cells. Furthermore, we found that active Cdc42 increased the total number of intracellular dense-core vesicles in the cells revealed by confocal microscopy. Based on these findings, we propose that Cdc42 induces the biogenesis of dense-core vesicles and regulates the number of exocytosis-competent dense-core vesicles in the neuroendocrine PC12 cells.

2. Materials and methods

2.1. Materials

Anti-chromogranin A (CgA) rabbit polyclonal antibody was from Prof. Kinji Inoue (Saitama University, Japan). Goat anti-rabbit Alexa Fluor 488 IgG was from Invitrogen (Eugene, OR, USA).

2.2. Plasmid constructions

A stop codon deleted monomeric red fluorescent protein (mCherry) fragment [8] was amplified by PCR and inserted into the BamHI-EcoRI site of pcDNA3.1(+) (Invitrogen) as a pcDNA 3.1(+)-mCherry. The full length of Cdc42 was amplified from Marathon-Ready mouse brain cDNA (Clontech) by PCR with the following pairs of oligonucleotides containing a restriction enzyme site (underlined): 5′-GAATTCATGCAGACAATTAAGTGTGTT-3′ and 5′-CTCGAGTCATAGCAGCACACACCTGCG-3′. The fragment was finally inserted into the pcDNA3.1(+)-mCherry vector, and named mCherry-Cdc42(WT). A constitutively-active form (G61L) and dominant-negative form (T17N) of Cdc42 were created by using conventional PCR techniques, and named mCherry-Cdc42(CA) and mCherry-Cdc42(DN), respectively. A stop codon deleted mCherry fragment was amplified by PCR again, and the amplified PCR fragment was inserted into the AgeI-BspEI site of the pEGFP-C1 vector (Clontech) replaced by EGFP with same frame as a pmCherry-C1 vector. The Cdc42/Rac-interactive binding (CRIB) domain of neuronal Wiskott–Aldrich syndrome protein (N-WASP) [4] was amplified from a PC12 cell cDNA library by PCR with the following pair of oligonucleotides containing restriction enzyme site (underlined) and a stop codon (boldface): 5′-GAATTCTGCTCCAAATGGTCCCAA-3′ and 5′-GCGGCGCC**TTA**TGCTTGCCTTCGGAG-3′. The fragment was finally inserted into the pmCherry-C1 vector, and named mCherry-CRIB. Neuropeptide Y-tagged Venus (NPY-Venus) was prepared as described previously [9]. GFP-Tuba and mCherry-Tuba were generous gift from Prof. Frank B. Gertler (Massachusetts Institute of Technology, USA) [10].

2.3. Cell culture

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum, 10% horse serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin, at 37 °C under 5% CO_2. For live-cell imaging, PC12 cells were plated onto poly-L-lysine-coated coverslips, and the cells were co-transfected with 3 μg NPY-Venus and 3 μg of mCherry-Cdc42(WT), mCherry-Cdc42(CA), or mCherry-Cdc42(DN) vectors using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

2.4. Total internal reflection fluorescence microscopy

Total internal reflection fluorescence (TIRF) imaging was performed in modified Ringer buffer at 37 °C (RB: 130 mM NaCl, 3 mM KCl, 5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4). High-KCl stimulation was achieved by perfusion with RB-containing 70 mM KCl (the NaCl concentration was reduced to maintain the osmolality). We mounted a high numerical aperture objective lens (Plan Apochromatic, 100×, NA = 1.45, infinity corrected, OLYMPUS) on an inverted microscope (IX71, OLYMPUS) and introduced an incident light for total internal reflection illumination through the high numerical aperture objective lens via a single mode optical fiber and illumination lens (IX2-RFAEVA-2, OLYMPUS). To observe the NPY-Venus fluorescence image, we used a diode-pumped solid-state 488 nm laser (HPU50100, 20 mW, Furukawa Electronic) for total internal fluorescence illumination and a band pass filter (HQ535/30m, Chroma) as an emission filter. Images were acquired every 300 ms or otherwise as indicated.

2.5. Immunofluorescence

PC12 cells were plated onto poly-L-lysine-coated coverslips and transfected with mCherry-Cdc42(WT), mCherry-Cdc42(CA), mCherry-Cdc42(DN), pmCherry-Tuba, or CRIB-mCherry as described above.

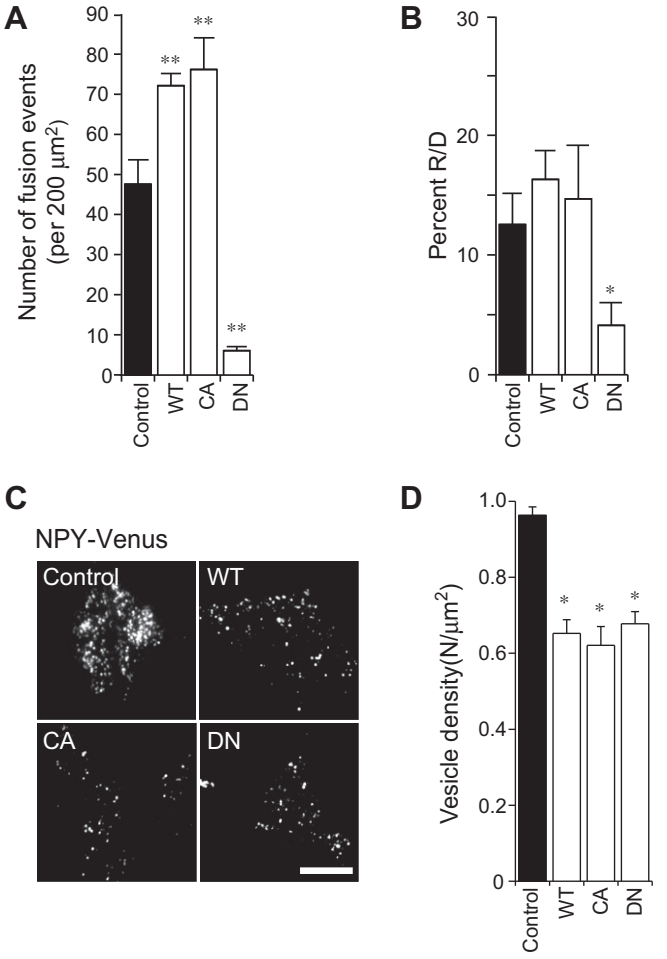

Fig. 1. Cdc42 increased the number of fusion events. (A) The number of fusion events for NPY-Venus during 5 min stimulation in control (Control), wild-type Cdc42 (WT), constitutively-active Cdc42 (CA), or dominant-negative Cdc42 (DN) overexpressing PC12 cells ($n = 15$ cells in each). (B) Percentage of NPY-Venus release events in PC12 cells overexpressing Cdc42 or its mutants during the 5 min stimulation determined from the number of plasma membrane-docked vesicle before stimulation under TIRF microscopy ($n = 15$ cells in each). (C) Typical TIRF images of plasma membrane-docked NPY-Venus vesicles observed in each cells. Scale bar = 10 μm. (D) The density of the plasma membrane-docked NPY-Venus vesicles was measured by counting the vesicles in each image ($n = 7$ cells in each). Data shown as mean values ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$ in comparison with the control, respectively.