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Vesicular nucleotide transporter is involved in ATP storage of secretory lysosomes in astrocytes

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ABSTRACT

Recent studies have suggested that astrocytes release gliotransmitters (i.e., ATP, L-glutamate, D-serine, and peptide hormones) and participate actively in synaptic functioning. Although ATP release from astrocytes modulates the activity of neurons, the mechanisms regulating the ATP release from astrocytes and the source of ATP in astrocytes are not well understood. Recently a vesicular nucleotide transporter (VNUT)/solute carrier family 17, member 9 (SLC17A9) has been identified as a mediator of the active accumulation of ATP into vesicles. Here we show by immunocytochemical analysis under confocal microscope and live cell imaging under total internal reflection fluorescence microscope that lysosome-associated VNUT is responsible for ATP release in astrocytes. VNUT was expressed in both primary cultured cortical astrocytes and glioma cell line C6 cells, and mainly localized on lysosome in the cells. We found that VNUT-associated secretory lysosomes do not fully collapse into the plasma membrane after lysosomal exocytosis. We also found that inhibition of VNUT function by Evans Blue decreased ATP uptake into secretory lysosomes. Depletion and inhibition of endogenous VNUT by small interference RNA and Evans Blue, respectively decreased the amount of ATP release from the cells, whereas overexpression of VNUT increased it. Taken together, these findings indicate that the participation of VNUT in ATP storage in secretory lysosomes during lysosomal exocytosis of ATP from astrocytes.

1. Introduction

Astrocytes are large population of cells in the central nervous systems (CNS). Recent studies have demonstrated that astrocytes respond to neurotransmitters and release various gliotransmitters, including glutamate [1], D-serine [2], ATP [3], and peptide hormones [4]. Extracellular ATP released from astrocytes can trigger inter-glia propagation of Ca^{2+} waves [5] and induce neuronal hyperpolarization [6]. Moreover, previous papers have suggested that astrocyte-derived ATP can modulate synaptic efficiency and plasticity in neurons [7,8]. Although ATP release from astrocytes modulates the activity of neurons, the mechanisms regulating the ATP release from astrocytes are not well understood.

The Ca^{2+}-regulated or unregulated ATP release has been reported. The release of ATP from astrocytes is reduced by application of inhibitors for connexin hemichannels [9], ATP-binding cassette transporters [10], and volume-regulated anion channels [11], suggesting that the involvement of multiple pathways in the release of ATP from astrocytes without Ca^{2+} regulation. However, these pathways are favored in conditions such as ischemia, traumatic injury, and seizure activity [12]. The Ca^{2+}-regulated ATP release from astrocytes has been suggested to utilize a similar mechanism with Ca^{2+}-regulated exocytosis of dense-core vesicles or synaptic-like vesicles. In fact, the release of ATP is dependent on the increase of intracellular Ca^{2+} concentration [13], and astrocytes possess various types of secretory vesicles, including synaptic-like vesicles, dense-core vesicles, and secretory lysosome [14]. Recent study has reported that secretory lysosomes are the major vesicular compartment contributing to Ca^{2+}-regulated ATP exocytosis from cortical astrocytes [15]. On the other hand, a vesicular nucleotide transporter (VNUT)/solute carrier family 17, member 9 (SLC17A9), a novel member of an anion transporter family, has been identified as a mediator of the active accumulation of ATP within vesicles [16]. Therefore, we focused on the role of VNUT on Ca^{2+}-regulated ATP release from secretory lysosome in astrocytes.

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In present study, to clarify the role of VNUT on ATP release from astrocytes, we examine the localization of VNUT in astrocyte and monitor the secretion and uptake of ATP in VNUT-associated vesicles. We found that VNUT is mainly localized in lysosome in both primary cultured cortical astrocytes and glioma cell line C6 cells. The VNUT-associated lysosome showed no change of the fluorescence intensity after application of calcium ionophore, A23187, to C6 cells, reflecting the VNUT-associated lysosome stay on the plasma membrane after exocytosis. Inhibition of VNUT function decreases the uptake of ATP in secretory lysosomes and the amount of ATP release. Based on these findings, we propose that ATP is stored into secretory lysosome by VNUT, and lysosomal exocytosis is evoked by Ca^{2+} elevation in the cells under new mechanism distinct from exocytosis of either dense-core vesicle or synaptic-like vesicles.

2. Materials and methods

2.1. Materials

Anti-vesicular nucleotide transporter (VNUT) rabbit polyclonal antibody was from Medical & Biological Laboratories (MBL, Nagoya, Japan). Anti-vesicular glutamate transporter 1 (VGLUT1) mouse monoclonal antibody was from Chemicon International Inc. (Temecula, CA, USA). Anti-lysosomal-associated protein-1 (LAMP-1) mouse monoclonal antibody was from Santa Cruz Biotechnology (Santa-Cruz, CA, USA). Alexa 568-conjugated anti-rabbit IgG goat antibody, Alexa 568-conjugated anti-mouse IgG goat antibody, LysoTracker, and Texas Red-dextran were from Invitrogen (Eugene, OR, USA). 2′-/3′-*O*-(N′-methylanthraniloyl) adenosine-5′-*O*-triphosphate (MANT-ATP) was from AnaSpec (Fremont, CA, USA). To knockdown endogenous VNUT expression, we used Dicer-substrate RNAs (DsiRNAs) against rat VNUT mRNA (5′-GGUGAGUAUUCGAGAGAAUGUCACGAA-3′) and matched DsiRNA negative controls (MBL).

2.2. Plasmid construction

pmCherry-N1, N3, and pVenus-N1 vector was created by replacing EGFP in pEGFP-N1 and N3 vector with mCherry and Venus, respectively. Mouse VNUT was amplified from a mouse spleen cDNA by PCR. The DNA fragment of VNUT was finally inserted into the pEGFP-N3 (Clontech) and pmCherry-N3, and named VNUT-EGFP and VNUT-mCherry, respectively. Mouse cathepsin D (clone ID I920196K11) was purchased from DNAFORM (Kanagawa, Japan). The DNA fragment of cathepsin D was finally cloned into pmCherry-N1 and pVenus-N1 vector, and named Cathepsin D-mCherry and Cathepsin D-Venus, respectively. To localize the fluorescent protein inside of vesicles, we utilized the method on VGLUT1-associated vesicles [17]. Briefly, the mCherry surrounded by N-terminus linker STSGGSGGTGGS and C-terminus linker GGTGGTGGSGGTG was inserted between Gly-99 and Gly-100 of mouse VNUT, and named VNUT-intra-mCherry. Other expression plasmids, including neuropeptide Y (NPY)-Venus and NPY-mCherry were prepared as described previously [18,19].

2.3. Cell culture and transfections

Primary astrocytes were made from Sprague–Dawley rat cerebral cortices taken at postnatal day 1. Astrocyte-enriched cultures were made following the method described previously [20]. Primary astrocytes were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). The culture medium was changed at day 3 *in vitro* (DIV3) and DIV6. Rat glioma cell line C6 cells were cultured in the same culture medium described above. The primary cortical astrocytes and C6 cells were plated onto poly-L-lysine coated 35 mm glass-based dishes and maintained in an incubator at 37 °C with 95% air and 5% CO_2. The primary astrocytes and C6 cells were transfected with 3 μg of plasmids using LipopfectAMINE 2000 Reagent (Invitrogen) according to the manufacturer's instruction.

2.4. Immunofluorescence

VNUT-EGFP- or NPY-Venus-transfected primary cortical astrocytes and VNUT-EGFP-transfected C6 cells were fixed with 2% paraformaldehyde (Wako Pure Chemicals Industries, Ltd. Osaka, Japan) in phosphate-buffered saline (PBS) at room temperature (RT) for 20 min. The cells were permeabilized with 0.1% Triton X-100 for 2 min and blocked with the blocking buffer (1% BSA and 0.1% Triton X-100 in PBS) for 1 h. The cells were then immunostained with either anti-VNUT rabbit polyclonal antibody (1/100 dilution), anti-VGLUT1 mouse monoclonal antibody (1/100 dilution), or anti-LAMP-1 mouse monoclonal antibody (1/200 dilution) for 1 h followed by incubation with either goat anti-rabbit Alexa Fluor568 antibody (1/3,000 dilution) or goat anti-mouse Alexa Fluor 568 antibody (1/3,000 dilution) for 1 h at RT. VNUT-EGFP-transfected primary cortical astrocytes or C6 cells were loaded with LysoTracker for 1 h at 37 °C. VNUT-EGFP-transfected C6 cells were loaded with either Texas Red-dextran in DMEM (10 μg/ml) for 1 h at 37 °C or MANT-ATP in DMEM (50 μM) for 5 h at 37 °C. Confocal images were obtained using a Nipkow-disk type confocal microscope (CSU-10, Yokogawa, Tokyo, Japan). The images of the cells were analyzed with MetaMorph software (Molecular Devices, Sunnyvale, CA, USA).

2.5. Total internal reflection fluorescence microscopy

To observe the exocytotic response of VNUT-mCherry-, VNUT-intra-mCherry-, and cathepsin D-Venus-overexpressing cells, we used a total internal reflection fluorescence (TIRF) microscope essentially as described previously [19,21]. The TIRF imaging was performed at 37 °C in an artificial cerebrospinal fluid (αCSF: 140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4). Stimulation was achieved by perfusion with either 10 μM A23187-containing αCSF, 300 μM ATP-containing αCSF, or 1 mM L-glutamate-containing αCSF. To analyze the TIRF imaging data, exocytotic responses were selected manually, and the average fluorescence intensity of individual vesicle in a 0.7 μm × 0.7 μm square placed over the vesicle center was calculated. Data are reported as means ± SEM of at least five individual experiments.

2.6. RNA interference of VNUT and ATP release assay

C6 cells cultured in 6-well plate were transfected with either 20 pmol/μl VNUT DsiRNA or 20 pmol/μl matched DsiRNA negative controls using X-treme GENE HP DNA Transfection Reagent (Roche Applied Science, Basel, Switzerland) according to the manufacturer's instructions. Two days after transfection, C6 cells were bathed with 1 mL of αCSF or 10 μM A23187-containing αCSF for 5 min at 37 °C. The supernatant was collected and the amount of ATP in the collections was measured by using luciferin-luciferase ATP assay kit (TOYO-ink, Tokyo, Japan).

2.7. RNA isolation and RT-PCR analysis

Total RNA from C6 cells was prepared with Xprep RNA Mini Kit (Philekorea, Daejeon, Korea) and treated with RNase-free DNase (Promega, Madison, WI, USA). RNA (1 μg) was reverse-transcribed

using High Capacity cDNA Reverse Trascription kit (Applied Biosystems, CA, USA). For PCR amplification of a vesicular nucleotide transporter/solute carrier family 17, member 9 (VNUT/SLC17A9; NM_001108613), the forward primer 5′-AGGTCATCTTGCTGTCAGCC-3′ and the reverse primer 5′-AAGGATCTCTCGCTCTCCTG-3′ were used; and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, NM_017008), the forward primer 5′-CCATCACCATCTTCCAGGAG-3′ and the reverse primer 5′-TTCAGCTCTGGGATGACCTT-3′ were used.

2.8. Statistics

Data are reported as the mean ± SE. Means were compared by ANOVA followed by a Newman–Keulus test or a Dunnett's test using Graph Pad Prism software (Graph Pad software, La Jolla, CA, USA).

3. Results

3.1. VNUT is localized on lysosome in both cultured cortical astrocytes and C6 cells

To examine the cellular distribution of a vesicular nucleotide transporter (VNUT)/solute carrier family 17, member 9 (SLC17A9) in astrocytes, we carried out an immunocytochemistry with VNUT-EGFP overexpressed primary cortical astrocytes (Fig. 1A) and C6 cells (Fig. 1B). Overexpression of VNUT-EGFP in both primary cortical astrocytes and C6 cells produced a punctate pattern of fluorescence. VNUT-EGFP-associated vesicles are well colocalized with anti-VNUT antibodies (90.2 ± 9.4% for $n = 6$ cells, (Fig. 1A), and 93.4 ± 5.4% for $n = 5$ cells, Fig. 1B), indicating that VNUT–EGFP can be used for as a surrogate marker for VNUT-associated vesicles. The VNUT-associated vesicles showed a subtle colocalization with both synaptic-like vesicle marker, vesicular glutamate transporter 1 (VGLUT1) (11.2 ± 5.2% for $n = 5$ cells, (Fig. 1A), and 8.3 ± 3.2% for $n = 7$ cells, Fig. 1B), and dense-core vesicle marker, neuropeptide Y (NPY) (12.0 ± 10.3% for $n = 4$ cells, (Fig. 1A), and 11.2 ± 6.5% for $n = 5$ cells, Fig. 1B), while VNUT protein mainly existed on lysosome revealed by costained with lysosomal marker, LysoTracker (64.5 ± 5.3% for $n = 4$ cells, (Fig 1A), and 83.1 ± 11.4% for $n = 5$ cells, Fig. 1B). Since VNUT–EGFP-associated vesicles are well colocalized with LysoTracker, we further confirmed the localization of VNUT by other lysosomal maker in C6 cells. We found that the lysosomal markers, including cathepsin D (lysosomal protease) (93.3 ± 1.7% for $n = 3$ cells, Fig. 2A), Texas Red-dextran (accumulated in lysosome) (92.2 ± 4.8% for $n = 3$ cells, Fig. 2B), and lysosomal-associated protein-1 (LAMP-1) (95.6 ± 4.1% for $n = 3$ cells, Fig. 2C) were well colocalized with VNUT–EGFP. These results suggest that VNUT existed on lysosomal membrane and could function as ATP transporter in astrocytes.

3.2. Kinetics of lysosomal exocytosis is distinct from dense-core vesicle exocytosis

To analyze the kinetics of the exocytosis for secretory lysosomes in C6 cells, we overexpressed cathepsin D-Venus, VNUT-mCherry, or VNUT-intra-mCherry and monitor the fluorescence intensity change after stimulation under TIRF microscope (Fig. 3A). After Ca^{2+} elevating stimulation by calcium ionophore, A23187 (10 μM), some cathepsin D-Venus fluorescent spots brightened and subsequently dimmed (Fig. 3B), implying that the secretory lysosome cargoes were released into the extracellular space by Ca^{2+} elevation, consistent with dense-core vesicle exocytosis [19]. In contrast, VNUT-mCherry fluorescence remained nearly constant after stimulation (Fig. 3C), reflecting VNUT remained associated with the secretory lysosome and failed to spread into the plasma membrane, while a dense-core vesicle-associated protein (e.g., VAMP2) spread into plasma membrane

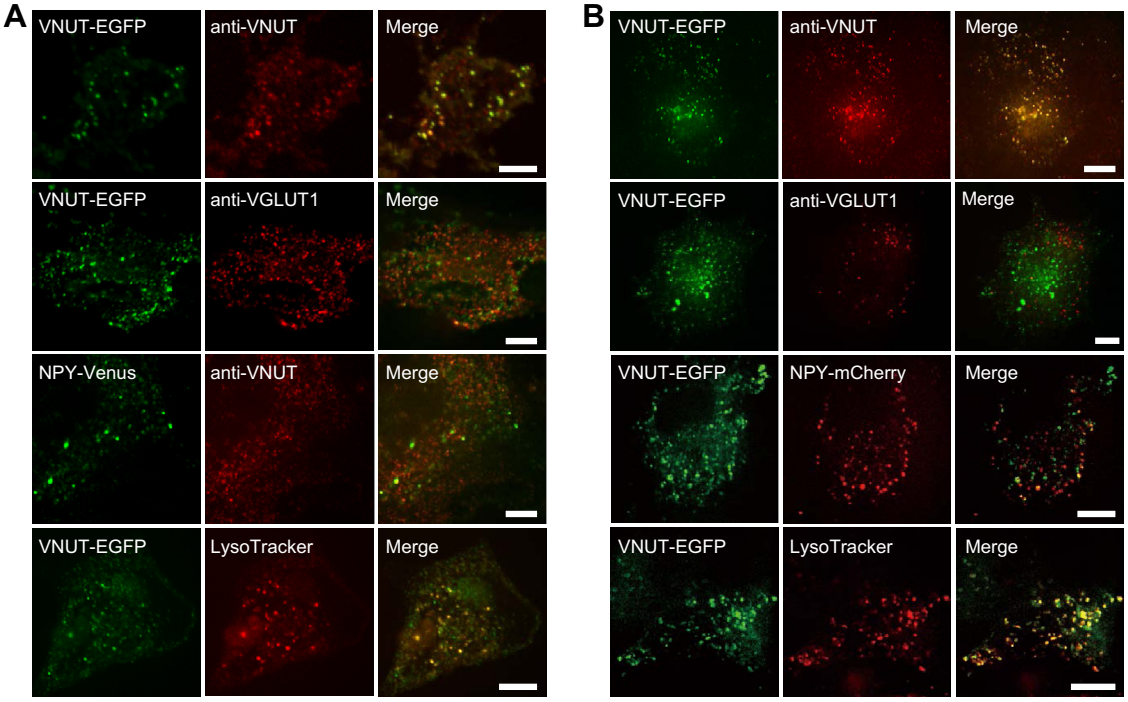

Fig. 1. VNUT largely colocalized with lysosomal marker in both primary astrocytes and C6 cells. (A) Cultured primary astrocytes expressing VNUT–EGFP were immunostained with anti-VNUT antibody (top), anti-VGLUT1 antibody (upper middle) or LysoTracker (bottom). Primary astrocytes expressing NPY-Venus was immunostained with anti-VNUT antibody (lower middle). Scale bar = 10 μm. (B) C6 cells expressing VNUT–EGFP were labeled with anti-VNUT antibody (top), anti-VGLUT1 antibody (upper middle), or LysoTracker (bottom). C6 cells were co-transfected VNUT–EGFP and NPY-mCherry (lower middle).Scale bar = 10 μm.

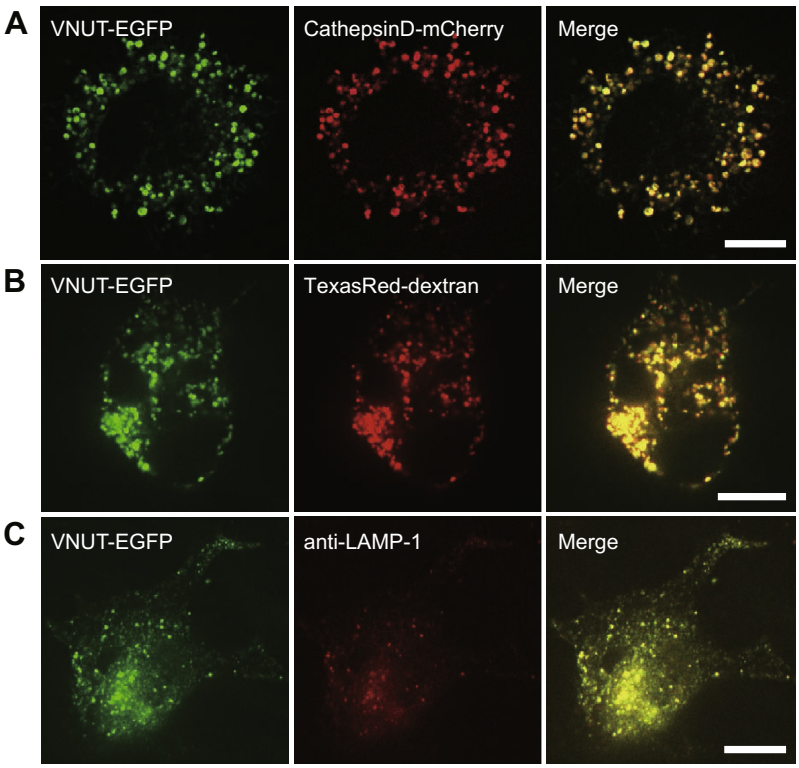

Fig. 2. VNUT colocalized with various lysosomal markers in C6 cells. The C6 cells expressing VNUT–EGFP were labeled with cathepsin D-mCherry (A), Texas Red-dextran (B), and anti-LAMP-1 antibody (C). Scale bar = 10 μm.

during exocytosis [19]. To confirm whether VNUT-associated vesicles create the fusion pore for releasing cargoes, we monitored the fluorescent intensity change of VNUT-intra-mCherry fused mCherry to a first luminal/external loop of VNUT (Fig. 3A). Application ofA23187 (10 μM) induced an increase of fluorescence intensity of VNUT-intra-mCherry and subsequently dimmed (Fig. 3D), suggesting that VNUT-associated secretory lysosome release their cargoes during exocytosis. To investigate whether lysosomal exocytosis is induced by physiological stimuli (i.e., ATP and L-glutamate), we counted the number of fusion events with other stimuli visualized by cathepsin D-Venus under TIRF microscope. Application of either ATP (300 μM), L-glutamate (1 mM), or A23187 (10 μM) induced the lysosomal exocytosis (Fig. 3E). Since it has been suggested that released ATP from lysosomes and dense-core vesicles in astrocytes induced glutamate release form synaptic-like vesicles in the astrocytes, and vice versa [22,23]. Thus, the different types of secretory vesicles (i.e., dense-core vesicles, synaptic-like vesicles, and secretory lysosomes) exocytosed from the astrocytes might cooperatively modulate the neuron-glia interaction.

3.3. VNUT transports ATP into the secretory lysosome

To investigate whether VNUT acts as an ATP transporter on secretory lysosome, we incubated C6 cells with 2′-/3′-*O*-(N′-methylanthraniloyl) adenosine-5′-*O*-triphosphate (MANT-ATP), a fluorescent ATP analog for checking ATP uptake. Loading of MANT-ATP to the cells produced a punctate pattern of fluorescence (Fig. 4A). MANT-ATP-containing vesicles were well colocalized with VNUT-mCherry (71.7 ± 7.5% for $n = 4$ cells, Fig. 4A). When we pre-incubated C6 cells with Evans Blue (100 μM), pharmacological inhibitor for VNUT, the uptake of MANT-ATP were dramatically inhibited (Fig. 4B). These results suggest that ATP in secretory lysosome was accumulated via VNUT.

To examine whether VNUT is responsible for the amount of ATP release from astrocytes, we measured the released ATP by luciferin-luciferase ATP assay (Fig. 4D). Application of A23187 (10 μM) to the cells induced ATP release, and the amount of released ATP is increased by overexpression of VNUT. Moreover, the amount of released ATP by A23187 stimulation was returned to basal level by silence of VNUT (Fig. 4C) by VNUT DsiRNA or inhibition of VNUT function by Evans Blue (100 μM). This effect was validated by negative control DsiRNA using DsiRNA with scrambled sequence (Fig. 4C and D). These results strongly indicate that astrocytes release ATP from VNUT-associated secretory lysosome.

4. Discussion

In the present study, we have shown that VNUT-associated vesicles were well colocalized with lysosome. Secretory lysosomes release their cargoes such as ATP respond to Ca^{2+}-elevating stimulation. Although several studies have shown that ATP release from astrocytes was from lysosomes which is consistent with our present findings [15,22], some previous studies have shown that exocytotic release of ATP from astrocytes was from dense-core vesicles [24]. This discrepancy could be ascribed to existence of the multiple ATP storage sites in astrocytes (i.e., lysosomes and dense-core vesicles). In fact, we found that a few VNUT-associated vesicles are not colocalized with LysoTracker (Fig. 1A, B bottom), and some of MANT-ATP-containing vesicles are also not colocalized with VNUT-associated vesicles (Fig. 4A). These findings suggest that the mechanisms of ATP release could also include exocytosis, although the nature of ATP released from astrocytes may vary with various physiological and pathological conditions [12,13,24]. Thus, further studies are required to elucidate the mechanisms by which astrocytes release ATP in response to distinct stimuli, as this will further clarify the mechanisms underlying the neuron-glia interaction.

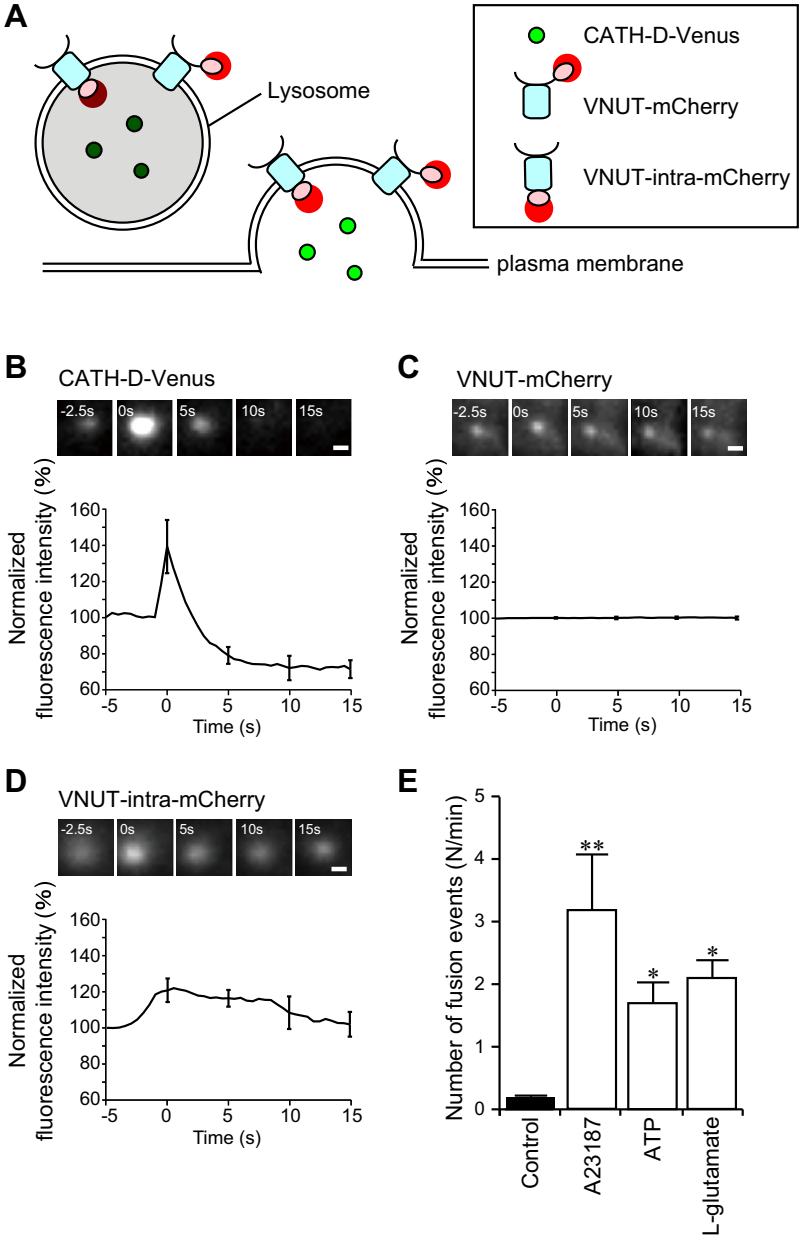

Fig. 3. VNUT remains in lysosomal membrane during exocytotic events. (A) Schematic drawing of various types of lysosomal markers used in the present study: VNUT-mCherry (red, outside of lysosome), VNUT-intra-mCherry (red, inside of lysosome) and cathepsin D (CATH-D)-Venus (green, lysosome cargo). (B, C, D top) Typical sequential images of a single CATH-D-Venus- (B), VNUT-mCherry- (C) and VNUT-intra-mCherry- (D) expressed lysosomes observed by TIRF microscopy after application of 10 μM A23187. Scale bars = 1 μm. (B, C, D bottom) Time course of fluorescence intensity changes measured in the center of CATH-D-Venus- (B), VNUT-mCherry- (C) and VNUT-intra-mCherry- (D) expressed lysosomes. The mean fluorescent intensity was normalized to 100%. (E) The number of cathepsin D exocytotic events during application of various stimuli (i.e., A23187, ATP, and L-glutamate) in 14 min. Data are shown as mean ± SE, $^{*}p < 0.05$, $^{**}p < 0.01$ ($n = 4$ trials in each condition). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

We found that the fluorescence intensity of VNUT-mCherry was stable during the lysosomal exocytosis, indicating that secretory lysosomes maintain their spherical structure for quite a long time (probably minutes) after fusion to plasma membrane (Fig. 3D). This phenomenon is quite distinct from the behavior of dense-core vesicles or synaptic vesicles during fusion events, which easily spread into the plasma membrane. Recently, vesicular acetylcholine transporter (VAChT) diffuses into the plasma membrane only several hundred nanometers [25]. This limited diffusion of VAChT to the plasma membrane is regulated by dense clathrin network which corrals over VAChT, and organizes and maintains the shape of VAChT-associated vesicles during exocytosis. We thus speculate that dense clathrin network could also corral over VNUT and recycle VNUT-associated secretory lysosome rapidly. Further studies are required to clarify whether dense clathrin network regulates VNUT-associated lysosome recycling.

In summary, we have investigated the intracellular distribution of VNUT and the role of VNUT on ATP release from astrocytes using confocal microscopy and live cell TIRF microscopy, respectively. Our findings provide direct evidence that VNUT is a key element of ATP storage in lysosome and exocytosis of ATP from lysosome in astrocytes. We propose that secretory lysosomes may attach and stay to the plasma membrane after fusion. Therefore, we conclude that a 'kiss and stay' process occurs during lysosomal exocytosis from VNUT-associated vesicles. Elucidating the precise molecular mechanism of endocytotic proteins (i.e., clathrin)

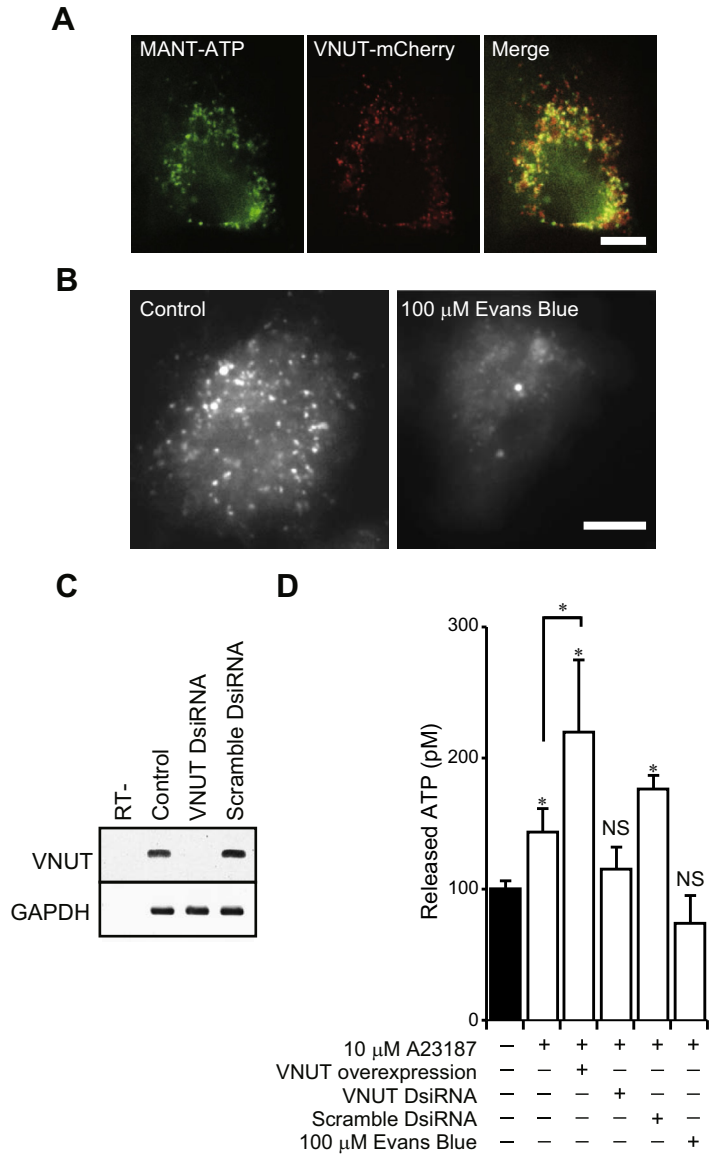

Fig. 4. VNUT mediates ATP secretion. (A) Uptake of MANT-ATP was colocalized with VNUT-mCherry-expressed lysosomes in C6 cells. Scale bar = 10 μm. (B) The accumulation of MANT-ATP was inhibited by application of Evans Blue (100 μM). Scale bar = 10 μm. (C) RT-PCR analysis of the effect of VNUT DsiRNA on the expression of VNUT in C6 cells. The expression of VNUT mRNA was reduced by specific VNUT DsiRNA. (D) The amount of secreted ATP after application of 10 μM A23187 in C6 cells, VNUT overexpressing cells, VNUT-depleted cells, and VNUT inhibitor treated cells was measured by luciferin-luciferase assay. Data are shown as mean ± SE, $^{*}p < 0.05$ (n = at least 5 trials in each condition).

recruitment to the fusing lysosome may provide further valuable insight into this form of exocytosis-coupled endocytosis in lysosome.

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