Suppression of transmitter release by Tat HPC-1/syntaxin 1A fusion protein

Tomonori Fujiwara a, Tetsuo Yamamori b, Kimio Akagawa a.*

a Department of Physiology, Kyorin University School of Medicine, Shinkawa 6-20-2, Mitaka, Tokyo, 181-8611 Japan

b Division of Speciation Mechanisms, National Institute for Basic Biology, 38 Nishigonaka, Myodaijicho, Okazaki, Aichi, Japan

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Abstract

It has been reported that the fusion protein with the protein transduction domain (PTD) peptide of HIV-1 Tat protein can be internalized through the cell membrane of intact cells, although the exact mechanism is unknown. In this report, we investigated whether this new method could be used for the molecular analysis of exocytosis via HPC-1/syntaxin 1A, which plays an important role in transmitter release. When applied to PC12 cells, Tat PTD fusion proteins were rapidly internalized into most cells. In order to show that the internalized protein remained biologically active, the H3 domain of HPC-1/syntaxin 1A was fused to Tat PTD (Tat-H3). Transmitter release in PC12 cells was suppressed by Tat-H3 treatment. These results indicate that the Tat fusion protein is a useful tool for analyzing the process of transmitter release. ß 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

HPC-1/syntaxin 1A plays an important role in exocytosis [1–3]. This protein binds to synaptosomal-associated protein of 25 kDa (SNAP-25) and vesicle-associated membrane protein (VAMP2), forming a stable complex called the SNARE complex (soluble N-ethylmaleimide sensitive fusion protein attachment protein receptor complex) [4]. It is thought that assembly or disassembly of this stable complex occurs during the process of intracellular membrane fusion. It is well known that one of the α-helical structural regions of HPC-1/syntaxin 1A, called the H3 domain, contributes to SNARE complex formation. Although it is also known that this domain is essential for binding to other SNAREs [5], the exact mechanism of transmitter release via SNARE is not yet clear.

In order to understand the intracellular molecular mechanism of exocytosis in detail, many experiments using living cells have been performed. For example, digitonin and streptolysin-O permeabilized cells have been used to introduce macromolecules, including proteins, into cells [6]. However, it is thought that permeabilized cells may differ from intact cells, since the cells become leaky or fragile. During microinjection into an intact cell, it is difficult to control the concentrations of the injected reagents in the cell. Recently, it was reported that the β-galactosidase fusion protein with the protein transduction domain

* Corresponding author. Fax: +81-42-47-48-01; E-mail: akagawak@kyorin-u.ac.jp

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(PTD) peptide of HIV-1 Tat protein could be internalized through the cell membranes of intact cells [7,8]. Tat PTD peptide chemically crosslinked with horseradish peroxidase (HRP) or IgG has also been internalized into many types of cells [9,10]. However, this has not been used to analyze the process of exocytosis.

This report shows that the Tat PTD method using Tat-H3 fusion protein can be a useful tool for the molecular analysis of the process of transmitter release. Furthermore, we show that the suppressive effect of Tat-H3 treatment is accelerated by prestimulation.

2. Experimental procedure

2.1. Antibodies

IgG was purified from normal rabbit serum with protein A Sepharose (Amersham Pharmacia Biotech). Anti-H3 antibody was raised against recombinant protein as reported [11]. The specificity of this antiserum was examined by immunoblotting with rat brain homogenate (data not shown). Anti-His tag antibody was purchased from Santa Cruz Biotechnology.

2.2. Plasmid construct

A double stranded oligonucleotide encoding Tat PTD peptide (GYGRKKRRQRRRG) was ligated with a green fluorescence protein (GFP) cDNA fragment. This fragment was subcloned into pPro Ex-1 vector (Gibco BRL). HPC-1/syntaxin 1A cDNA fragments were prepared by PCR. The PCR fragments encoding N265 (1–265), N201 (1–201), N97 (1–97), and H3 (202–265) were replaced with GFP cDNA from the Tat-GFP vector.

2.3. Protein expression and peptide conjugated protein preparation

All recombinant proteins were produced in Escherichia coli DH5α cells by induction with 1 mM IPTG. These recombinant proteins (His tag fusion proteins) were extracted with 8 M urea solution containing 100 mM NaCl and 20 mM HEPES (pH 8.0). The extracts were loaded on a Ni-agarose column. Recombinant proteins were eluted with buffer solution containing 200 mM imidazole. The purified recombinant proteins were desalted on a PD-10 column (Amersham Pharmacia Biotech) into Hanks’ balanced salt solution (HBSS) and applied to culture medium.

IgG was dissolved in 0.1 M borate buffer (pH 8.5) at 5 mg/ml and incubated with a 5–10-fold molar excess of sulfo succinimidyl-6-[3’-(2-pyridyldithio)-propionamido]hexanonate (slcSPDP Pierce) for 3 h at 25°C. After dialysis against phosphate-buffered saline (PBS), the reaction product was incubated with a 3-fold molar excess of Tat PTD peptide for 24 h at 4°C. Finally, the peptide conjugated IgG was dialyzed against HBSS.

2.4. Immunohistochemistry

PC12 cells were maintained in DMEM (Sigma) containing 5% fetal bovine serum and 5% horse serum [6]. For staining of internalized Tat-H3 into the cells, PC12h cells (1 × 10^5 cells/cm^2) were plated on a collagen coated cultured dish. Two days after plating the cells, 0.3 μM Tat-H3 or H3 was added to the culture medium. After 10 min incubation, the medium was washed out, and these cells were fixed with 4% paraformaldehyde (PFA) for 10 min, followed by 100% cold methanol treatment. These cells were further incubated with 0.1% Triton X-100 containing 4% PFA solution. These cells were then incubated with anti-His antibody. To visualize the internalized protein, DAB was used for a substrate of HRP conjugated anti-rabbit IgG and the cells were observed microscopically. Fluorescent of GFP was visualized with an inverted microscope.

2.5. ELISA

To study the time course of Tat fusion protein internalization, cells were cultured on collagen coated 96-well plates. Two days after plating the cells, 0.3 μM Tat-H3 was added to each well. After an appropriate incubation, the cells were fixed with 4% PFA solution. These cells were then incubated with anti-His antibody with or without 0.1% Triton X-100 containing 4% PFA solution. To detect internalized protein quantitatively, 3,3′,5,5′-tetramethyl-
benzidine (TMBZ) was used as a substrate for HRP conjugated anti-rabbit IgG. Each well was analyzed by densitometric measurement at 450 nm.

2.6. Immunoblotting

Tat-H3 or H3 treated PC12 cells were homogenized in PBS. After the nuclear pellet was removed by centrifugation, the supernatant was further centrifuged at 100,000×g for 1 h. The pellets (membrane fraction) were resuspended in a volume of PBS equal to that of the supernatant (cytosol fraction). These protein fractions were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by immunoblotting with the anti-H3 antibody.

2.7. Release assay

Two days before the experiments, PC12h cells (5×10^5 cells/well) were plated on collagen coated 24-well plates [12]. To measure noradrenaline (NA) release, PC12 cells were preloaded with 25 nM [3H]NA (Amersham Pharmacia Biotech) for 3 h. Then, the cells were incubated with Tat fusion protein. After an appropriate incubation, the culture medium was washed out. Then, the medium was replaced with HBSS four times every 5 min, and these fractions were collected to measure constitutive release. The cells were stimulated with 0.1 mM carbachol (CCh)/HBSS or high K+ solution (114 mM NaCl, 30 mM KCl, 1.25 mM KH2PO4, 1 mM MgSO4, 1.2 mM CaCl2, 2 mM NaHCO3, 10 mM glucose, 5 mM HEPES pH 7.4) for 5 min (stimulated release). After collecting the medium, the cells were lysed with 0.1 N HCl to measure the [3H]NA remaining in the cells. The amount secreted was calculated as a percentage of the total radioactivity given by the sum of the remaining and secreted [3H]NA. The amount of release with CCh or K+ stimulation was calculated as the difference between constitutive release and stimulated release.

Dopamine (DA) release was assayed by high performance liquid chromatography with an electrochemical detector [12]. The amount of secretion was calculated as a percentage of the total DA content. The amount of release with 0.1 mM CCh or K+ stimulation was calculated as the difference between constitutive release and stimulated release.

3. Results

3.1. Tat fusion protein was internalized into PC12 cells

In order to test whether Tat PTD mediated protein internalization occurred, PC12 cells were incubated with recombinant fusion protein. As seen in Fig. 1A, Tat-GFP was internalized into most PC12 cells within 10 min, but this was not observed with GFP treatment alone (Fig. 1B). PC12 cells were also incubated with Tat PTD fused to the H3 domain of HPC-1/syntaxin 1A (Tat-H3), which is known to bind to other SNARE proteins and to suppress transmitter
release in intact cells [13]. Immunohistochemical study showed that Tat-H3 was also rapidly internalized (Fig. 1C), but this was not observed with H3 treatment alone (Fig. 1D). Since this signal was not observed without permeabilization by Triton X-100 before incubation with the antibody (data not shown), it was thought that Tat-H3 was certainly internalized into the PC12 cell. Immunoblotting also revealed the presence of internalized Tat-H3 in both the cytosol and membrane fractions (Fig. 1E, lanes 1 and 2), but this was not observed with H3 treatment alone (Fig. 1E, lanes 3 and 4). These results clearly showed that Tat PTD mediated protein internalization occurred in PC12 cells.

We tried to study the time course of internalization in detail. As seen in Fig. 2, the internalized protein was detected within 5 min of addition of the fusion protein. This intracellular signal was not observed without Triton X-100 treatment. The conjugate of IgG chemically crosslinked with the PTD peptide was also rapidly internalized within 5 min, but IgG alone was not internalized. A similar result was also obtained for cultured hippocampal neurons (data not shown). Furthermore, this internalization was dose-dependent (data not shown). These results revealed that Tat PTD mediated internalization occurred rapidly in PC12 cells and neurons.

3.2. Suppression of transmitter release in Tat-H3 treated cells

To study whether the internalized proteins remained functional, PC12 cells were incubated with 0.3 μM Tat-H3 for 18 h and labeled with [3H]NA for 3 h. There was no difference in the [3H]NA uptake into Tat-H3 treated cells and Tat-GFP treated controls (data not shown). These cells were stimulated with high K+ for 5 min. The amount of [3H]NA release with Tat-H3 treatment was significantly lower than with Tat-GFP or H3 treatment (Fig. 3A). This suppressive effect was also observed in [3H]NA prelabeled cells before Tat-H3 treatment (Fig. 5A). The suppression of [3H]NA release was dose-dependent and the estimated concentration for half-maximum efficiency was about 0.2 μM. In order to test whether Tat-H3 treatment suppresses Ca2+-dependent transmitter release, PC12 cells were stimulated under Ca2+-free conditions. As shown in Fig. 3A, the amount of [3H]NA release with the Tat-H3 treatment was not significantly different from that with Tat-GFP or H3 treatment in Ca2+-free conditions. These results suggest that internalized Tat-H3 remained functional in PC12 cells.

We further examined whether another HPC-1/syntaxin 1A fragment affected transmitter release in PC12 cells. Treatment of Tat PTD fused with HPC-1/syntaxin 1A fragments other than H3 did not show significant effects on [3H]NA release (Fig. 3C), indicating that the suppressive effect was specific to the H3 domain. A similar result was also obtained with CCh stimulation (data not shown).

In order to examine the effect of endogenous transmitter release, DA release was measured in Tat-H3 treated PC12 cells. DA release from Tat-H3 treated PC12 cells was significantly lower than with Tat-GFP or H3 treatment (Fig. 3B). Under Ca2+-free conditions, DA release from the Tat-H3 treated cells was not significantly different from the release from Tat-GFP treated cells. These results indicated...
that Tat-H3 treatment suppressed Ca\(^{2+}\)-dependent DA release.

We next examined the effect of Tat-H3 on exocytosis with 0.1 mM CCh stimulation. As shown in Fig. 4A, the amount of \(^{3}H\)NA released from Tat-H3 treated PC12 cells was significantly lower than with Tat-GFP or H3 treatment. DA release with CCh stimulation in Tat-H3 treated PC12 cells was also significantly lower compared with Tat-GFP or H3 treatment (Fig. 4B).

### 3.3. Modification of the suppressive effect of Tat-H3 by prestimulation

As shown in Fig. 1B, Tat fusion protein was rapidly internalized. In order to study whether the suppressive effect of Tat-H3 treatment also occurred rapidly, we examined the time course of the Tat-H3 effect on the release of \(^{3}H\)NA. Although Tat-H3 was internalized within 5–10 min, suppression of \(^{3}H\)NA release was not observed until 30 min (Fig. 4B).
The maximal effect of Tat-H3 treatment appeared after 3 h. It was thought that the suppressive effect was caused by inhibition of SNARE complex formation by Tat-H3. Assuming that Tat-H3 only binds to newly synthesized or disassembled SNARE protein, the suppressive effect might be delayed after Tat-H3 internalization. According to this assumption, enhancement of SNARE complex disassembly could modify the time course of the Tat-H3 effect. To examine this possibility, Tat-H3 was washed out immediately after the first stimulation, and a second stimulation was applied (Fig. 5B). With the first stimulation, there was no difference in release between Tat-H3 treatment and the control. However, with the second stimulation, $[^{3}H]$NA release with Tat-H3 treatment was significantly lower than that of the control. This supported the hypothesis that the first stimulation enhances SNARE complex disassembly.
assembly and that Tat-H3 inhibits the assembly of new complexes during the second stimulation.

4. Discussion

In this study, we used a new protein introducing method mediated by Tat PTD. As seen in Fig. 1A,C, the Tat fusion protein was internalized with high efficiency. This internalization occurred rapidly (Fig. 2). Furthermore, the amount of internalized protein and the effect of Tat-H3 were dose-dependent (Fig. 5A). A similar result was observed in cultured hippocampal neurons (data not shown). These results showed that Tat PTD mediated protein internalization occurred in PC12 cells and neurons.

It has been reported that a HIV Tat protein is neurotoxic [14,15]. Although transmitter release was suppressed with 0.3 μM Tat-H3 treatment, application of Tat-GFP or Tat-H3 did not affect the viability of PC12 cells or cultured hippocampal neurons (data not shown). Accordingly, it was thought that the effective concentration of Tat-H3 used to suppress transmitter release was too low to cause cell toxicity. These results suggest that this protein introducing method is a useful tool for the molecular analysis of neurotransmitter release in intact cells.

The internalized Tat-GFP or Tat-IgG remained in cells at least 1 h after washing out the medium, but it became undetectable after 6 h (data not shown). The internalized protein was quickly lost, although it was not clear whether it leaked from the cell or was degraded in the cell. The suppressive effect of Tat-H3 treatment prolonged retention for at least 4 h after washing out the medium (Fig. 3). These results suggested that internalized Tat-H3 might bind to SNARE proteins in PC12 cells.

As seen in Fig. 3C, Tat HPC-1/syntaxin 1A fragment fusion proteins other than H3 did not affect transmitter release in PC12 cells. Although Tat-N265 contained the H3 domain, Tat-N265 treatment did not affect [3H]NA or DA release. This suggests that the N-terminus of HPC-1/syntaxin 1A contains a regulatory region [13,17]. Since Tat-N201 contains the N-terminus and lacks H3, the results of Tat-N201 treatment suggest that the N-terminal region of HPC-1/syntaxin 1A regulates the H3 domain.

However, further study is necessary to assess this hypothesis. Although the internalization occurred rapidly (Fig. 2), suppression of transmitter release by Tat-H3 treatment did not occur for 30 min (Fig. 5A). There are two explanations for this discrepancy. First, it is possible that the internalized Tat-H3 is not a cytosolic component and that Tat-H3 is internalized in endosomes and then gradually released into the cytosol. Second, Tat-H3 might be directly internalized into the cytosol, but since SNARE proteins form a stable complex in the cell, the internalized Tat-H3 might not affect complexes already formed. As shown in Fig. 5B, the suppression of transmitter release was observed 5 min after Tat-H3 treatment with previous stimulation. This result supports the latter hypothesis, since it has been reported that disassembly of formed SNARE complexes occurs during exocytosis [16]. If internalized Tat-H3 inhibited the assembly of new complexes, suppression of transmitter release would occur during the second stimulation.

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