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Prion Protein Does Not Interfere with SNARE Complex Formation and Membrane Fusion

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Abstract In prion disease, spongiform neurodegeneration is preceded by earlier synaptic dysfunction. There is evidence that soluble *N*-ethylmaleimide sensitive factor attachment receptor (SNARE) complex formation is reduced in scrapie-infected *in vivo* models, which might explain this synaptic dysfunction because SNARE complex plays a crucial role in neuroexocytosis. In the present study, however, it is shown that prion protein (PrP) does not interfere with SNARE complex formation of 3 SNARE proteins: syntaxin 1a, SNAP-25, and synaptobrevin. Sodium dodecyl sulfate-resistant complex formation, SNARE-driven membrane fusion, and neuroexocytosis of PC12 cells were not altered by PrP. Thus, PrP does not alter synaptic function by directly interfering with SNARE complex formation.

Keywords: prion, soluble *N*-ethylmaleimide sensitive factor attachment receptor (SNARE), membrane fusion, transmissible spongiform encephalopathy, synaptic transmission

Introduction

In prion disease, a portion of prion protein (PrP) converts from its normal conformation, PrP^{C}, to an insoluble and protease-resistant pathogenic conformation, PrP^{Sc} (1). This conformational change leads to infectious disorders affecting the brains of humans and animals. Prion diseases are characterized by a marked astrogliosis, spongiform degeneration, and neuronal loss in the brain (2).

While the exact mechanism that causes this neuronal cell death is still elusive, several studies have suggested that the accumulation of misfolded prion inhibits the formation of the soluble *N*-ethylmaleimide sensitive factor attachment receptor (SNARE) complexes involving synaptobrevin, syntaxin, and synaptosome-associated protein of 25 kDa (SNAP-25). SNARE proteins play an essential role in neurotransmitter release by mediating membrane fusion. Expression level of individual SNARE proteins and/or the extent of SNARE complex formation were associated with the presynaptic axon terminals changes which were induced by PrP (3-8).

Syntaxin 1a, SNAP-25, and synaptobrevin comprise the SNARE complex, which is a parallel 4 helical bundle (9,10). Syntaxin and synaptobrevin are transmembrane proteins with a single transmembrane helix, and are anchored to the presynaptic membrane and vesicular membrane, respectively. SNARE complex formation brings about close apposition of 2 membranes and facilitates membrane fusion. This membrane fusion eventually leads to fusion pore formation through which neurotransmitters are released (11). Thus, it seems quite obvious that impaired SNARE complex formation by PrP might impact synaptic function and contribute to or drive neuropathology, if PrP really interferes with SNARE interactions.

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Recently, quite contradictory results were presented from different groups. One study reported that expression of SNARE proteins and the interactions between them were altered in scrapie-infected GT-1 cells (12). SNARE complex formation was decreased in scrapie-infected GT-1 cells, which may be relevant to the exon terminal changes seen in prion-infected brains. In a contrasting study, SNARE complex formation was unaltered in another *in vivo* model, leading to the conclusion that the accumulation of PrP^{Sc} does not exert its effects by disrupting the formation of SNARE complexes (13). The major difference between these 2 contradictory results derives from the kind of detergents used to extract proteins from cells. Thus, the issue related to PrP-SNARE interaction needs to be clarified to obtain further insight into the neuropathological mechanism of prion disease.

We tested whether or not PrP interferes with SNARE function in 3 different ways. First, sodium dodecyl sulfate (SDS)-resistant SNARE core complex formation was measured in the presence or absence of PrP. Once SNARE complex is formed it is resistant to SDS (14). Thus, the appearance of a SDS-resistant complex band is often used as an indicator of SNARE complex formation. Second, the influence of PrP on SNARE-driven membrane fusion was assessed. In recent years, fusion of liposomes reconstituted with SNARE proteins has been widely used to investigate the mechanism of SNARE-mediated fusion and its regulation by other proteins (15-21). Third, the inhibition of neurotransmitter release from neuronal cells was assessed in the presence or absence of transfected PrP. None of our results supported reduced SNARE complex formation and direct interaction of PrP with SNARE proteins.

Materials and Methods

Materials 1-Palmitoyl-2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phospha-

tidylserine (DOPS), 1,2-dioleoyl-*sn*-glycero-3-phosphoserine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PS), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (rhodamine-PE) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). RPMI 1640, penicillin-streptomycin, horse serum, and fetal bovine serum (FBS) were purchased from Gibco-BRL (Grand Island, NY, USA). Biotreck kit was obtained from Stratagene (LaJolla, CA, USA). Triton X-100, 2-mercaptoethanol, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Protein expression and purification The C-terminal fragment spanning residues 90-231 of Syrian hamster prion protein was expressed in *Escherichia coli* BL21(DE3). The inclusion body of PrP was refolded as described previously (22). Refolded proteins were eluted with 20 mM Tris-HCl pH 7.4, 500 mM NaCl, and 500 mM imidazole. The solution was centrifuged to remove precipitates. Finally, salts were removed from solution using PD-10 desalting column (GE Healthcare, Buckinghamshire, UK). Full-length syntaxin 1a (SynF, amino acids 1-288), full-length synaptobrevin (VpF, amino acids 1116), SNAP-25 (amino acids 1206), soluble core domain of syntaxin 1a (SynH3), and soluble fragment of VAMP2 (VpS) were expressed as described elsewhere (21,23).

Reconstitution of SNARE proteins into membranes Large unilamellar vesicles (LUV) with a diameter of 100 nm were used in this study. A mixture of POPC and DOPS (molar ratio of 65:35) in chloroform was dried in a vacuum and resuspended in buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0) for a total lipid concentration of 50 mM. Protein-free LUV (approximately 100 nm in diameter) was prepared by extrusion through polycarbonate filters (Avanti Polar Lipids). Syntaxin 1a and SNAP-25 were mixed at room temperature for approximately 60 min to allow the formation of t-SNARE complex. Then, the t-SNARE complex was mixed with the prepared LUV at a 50:1 lipid/protein molar ratio. For the v-SNARE vesicles, 10 mM fluorescent liposomes containing POPC, DOPS, NBD-PS, and rhodamine-PE at a molar ratio of 62:35:1.5:1.5 were mixed with VAMP2 at a 50:1 lipid/protein ratio. The liposome/protein mixture was diluted 2 times, which brought the concentration of octyl glucoside below the critical micelle concentration. After dialyzing against dialysis buffer [25 mM HEPES, 100 mM KCl, 5%(w/v) glycerin, pH 7.4] at 4°C overnight to remove detergent, the sample was treated with Bio-Beads SM2 (Bio-Rad, Hercules, CA, USA) to eliminate any remaining trace amount of detergent. The solution was then centrifuged at 10,000×g for 30 min to remove protein and lipid aggregates. The final t-SNARE liposome solution contained approximately 2.5 mM lipids and 1.9 mg/mL of protein, and the v-SNARE liposome solution contained approximately1 mM lipids and 0.25 mg/mL of protein. The reconstitution efficiency was determined using SDS-polyacrylamide gel electrophoresis (PAGE). The amount of protein in the liposomes was estimated by comparing the band in the gel with that of the same protein of known concentration.

SNARE-driven membrane fusion assay To measure total lipid mixing, v-SNARE liposomes were mixed with t-SNARE liposomes at a ratio of 1:9. The final solution contained 1 mM lipids, with a total volume of 40 μL in the presence of prion protein. Fluorescence was measured at excitation and emission wavelengths of 465 and 530 nm, respectively. Changes in fluorescence were recorded with a Spectra Max M2 (Molecular Devices, Sunnyvale, CA, USA) fluorescence spectrophotometer. The maximum fluorescence intensity (MFI) was obtained by adding 0.1% Triton X-100. All lipid mixing experiments were carried out at 37°C.

PC12 cell culture PC12 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were plated onto poly-D-lysine-coated culture dishes and were kept in RPMI 1640 medium containing 100 μg/mL of streptomycin, 100 U/mL of penicillin, 2 mM L-glutamine, 10% heat-inactivated horse serum, and 5% FBS at 37°C in a 5% CO_2 incubator. The cell cultures were split once a week, and the medium was refreshed 3 times a week. The PC12 cells were treated with NGF (7S, 50 ng/mL, Invitrogen, Carlsbad, CA, USA) for 5 days prior to [^{3}H]-noradrenaline uptake and release.

Determination of [^{3}H]-noradrenaline release from detergent-permeabilized PC12 cells The amount of secreted [^{3}H]-noradrenaline was determined in PC12 cells. In brief, cells were grown in 12-well plates at a density of 4×10^5 cells/dish. After the cells adhered to the plates (20-24 hr), they were transfected with prion protein by transfection agents. After 5 hr, [^{3}H]-noradrenaline (1 μCi/mL) was treated at 37°C for 60 min in Krebs/HEPES solution (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15 mM HEPES-Tris, pH 7.4). The cells were washed 4 times to remove the unincorporated radiolabeled compound. The cells were then depolarized with a high-K^+ solution (115 mM NaCl, 50 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15 mM HEPES-Tris, pH 7.4) for 15 min to assess the stimulated release. Extracellular media were transferred to scintillation vials and were then measured by liquid scintillation counting. The quantity of [^{3}H]-noradrenaline release was calculated according to the following equation: quantity of [^{3}H]-noradrenaline release =(cpm of high K^+-stimulated sample – cpm of basal level release)/mg of protein.

Immunoblot Cells were washed twice with ice-cold phosphate-buffered saline (PBS) after treatment, and then lysed with RIPA buffer (Cell Signaling Technology, Beverley, MA, USA) directly in the plate wells after the removal of media. The cell lysates were obtained by centrifugation at 13,000×g for 15 min at 4°C. Protein concentrations were determined by the Bio-Rad protein assay kit using bovine serum albumin (BSA) as a standard. Equal amounts of protein (50 μg) from the cell lysates were dissolved in Laemmli's sample buffer (not boiled), electrophoresed (SDS-PAGE), and transferred to a nitrocellulose membrane. The membranes were blocked with PBS-0.1% Tween 20 (PBST buffer) containing 1% skim milk and 1% BSA for 1 hr at room temperature. Thereafter, the membranes were incubated overnight at

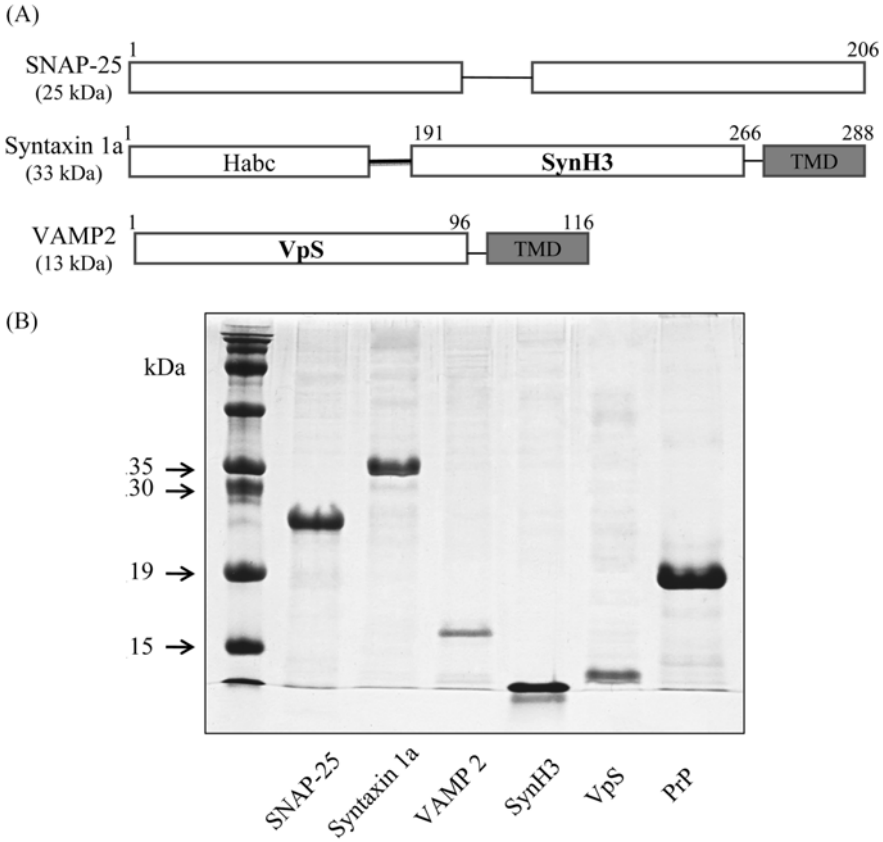

Fig. 1. Purified SNARE proteins and prion protein (PrP) used in this study. (A) Schematic diagram of SNARE proteins. Full-length SNAP-25, full-length syntaxin 1a, full-length synaptobrevin (VAMP2), soluble fragment of core domain of syntaxin 1a (SynH3), and soluble fragment of core domain of synaptobrevin (VpS) are shown. Their amino acid numbers are shown above the bar representing α-helix. TMD, transmembrane domain. (B) SDS-PAGE analysis of purified SNARE proteins and PrP.

4°C with a 1:1,000 dilution of monoclonal anti-SNAP-25 and anti-His tag antibodies. The membranes were washed 3 times with PBST and further incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. The membranes were then extensively washed 3 more times with PBST and developed by an enhanced chemiluminescence solution (ECL).

Results and Discussion

Effect of PrP on SDS-resistant SNARE complex formation To analyze the effect of PrP on SDS-resistant SNARE complex formation, soluble fragments of 2 SNARE proteins and 3 full-length proteins with transmembrane domains were prepared (Fig. 1). Syrian hamster $PrP_{90\text{-}231}$ (a PrP fragment containing amino acid residue 90-231) was also prepared (Fig. 1B). Equimolar amounts of soluble fragments of synaptobrevin (VpS) and SNAP-25, and soluble core domain of syntaxin 1a (SynH3) were mixed together in the presence or absence of PrP. After incubation at 25°C for 1 hr, the formation of SDS-resistant band was analyzed using SDS-PAGE (Fig. 2A). SNARE core complex formation was not inhibited even in the presence of 4-fold molar excess PrP. PrP has 1 disulfide bond, of which disruption might be involved in conformation change of PrP (24-29). Thus, we tested whether or not a reducing agent would bring about inhibitory effect of PrP on SNARE complex formation (right lanes of Fig. 2A). Reduced PrP did not inhibit SNARE complex formation. Furthermore, SNARE complex formation of full-length SNARE proteins was not affected by PrP regardless of reducing agent (Fig. 2B).

Inhibitory effect of PrP on SNARE-mediated membrane fusion SNARE proteins naturally function in membrane fusion (15,30,31). Thus, SNARE activity is often measured using reconstituted liposomes for their lipid mixing activity. If PrP really interferes with SNARE complex formation or interacts with individual SNARE proteins, SNARE-driven membrane fusion will be inhibited. Therefore, the inhibitory effect of PrP on SNARE proteins can be measured in functional level by exploiting this membrane fusion activity. After reconstituting SNARE proteins into appropriate vesicles (SynF and SNAP-25 in non-fluorescence liposome, and VpF in fluorescent liposome) both liposomes were mixed together. Then, membrane fusion driven by SNARE proteins was measured by reading the fluorescence increase as a function of time. Reconstituted SNARE proteins fused both membranes very well (circles in Fig. 3). Ten micomolar VpS (a soluble fragment of synaptobrevin without transmembrane domain) inhibited SNARE-driven membrane fusion very efficiently. VpS is thought to interfere with SNARE complex formation

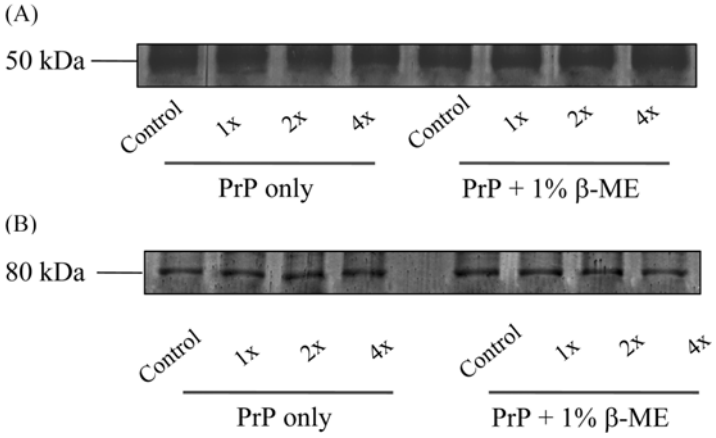

Fig. 2. Effect of PrP on SDS-resistant complex formation. Soluble core fragments of SNARE proteins (A) or full-length SNARE proteins (B) were mixed together in the presence or absence PrP. Left part of each gel was analyzed in the absence of reducing agent and right part of each gel was analyzed in the presence of 1% β-mercaptoethanol (β-ME).

between full-length SNARE proteins by competing with full-length synaptobrevin for its partner, t-SNARE complex (a binary complex composed of syntaxin and SNAP-25). If PrP interferes with SNARE complex formation or at least interacts with individual SNARE proteins, the increase in the fluorescence intensity should be apparently retarded comparably to VpS. However, we could not observe any inhibitory effect of PrP on membrane fusion even with 4-fold amount of PrP (Fig. 3). On the contrary, only a marginal increase of membrane fusion could be observed in the presence of PrP. This increase of fluorescence intensity was due to fusogenic activity of PrP itself. When both fluorescence and non-fluorescence liposomes were mixed together without SNARE reconstitution, PrP itself facilitated 2-3% maximal fluorescence intensity (gray bars in Fig. 3C). The presence of reducing agent did not alter the result (Fig. 3B). Thus, PrP does not inhibit SNARE-driven membrane fusion regardless of the redox condition.

Effect of PrP proteins in high K^+-treated PC12 cells

Finally, we tested whether or not transfection of PrP into neuronal cells inhibited SNARE complex formation using PC12 cells. Neurotransmitter release is reduced when SNARE complex formation is inhibited. For instance, synthetic peptides, botulinum neurotoxin, and plant extracts have been shown to inhibit neuroexocytosis by preventing SNARE complex formation (21,23). PC12 cells transfected with His6-PrP were incubated for further 4 hr, washed extensively, and measured for the amount of intracellular His6-PrP and SNARE complex using anti-His6-antibody and anti-SNAP-25-antibody, respectively. His6-PrP was well-transfected into PC12 cells but the amount of SNARE complex was not altered by PrP-transfection (Fig. 4A). Consequently, we could not measure any decrease in neurotransmitter release when PC12 cells were treated a high K^+ solution (Fig. 4B). The depolarization of these cells by the high K^+ concentrations (50 mM KCl) is known to result in the release of neurotransmitters such as noradrenaline, acetylcholine, and arachidonic acid (32,33). Verapamil, an L-type calcium channel blocker, inhibited neurotransmitter release very efficiently (34,35). Thus, we conclude that PrP does not inhibit SNARE complex formation in neuronal cells.

Prion diseases are neurological disorders triggered by the accumulation of a misfolded form of an endogenous, glycosyl phosphate inositide (GPI)-anchored cellular prion protein (1). The neurodegeneration is characterized by a marked astrogliosis, vacuolation, spongiform degeneration, and neuronal loss in the brain. There is evidence that synaptic dysfunction precedes the cell death that occurs at later stages (36,37). This earlier synaptic dysfunction is well-correlated with the findings of reduced expression of synaptic proteins, such as synaptophysin, SNAP-25, syntaxin 1A, and synapsin 1 (8,38). In the case of neurotransmitter release, the process is driven by SNARE complex formation composed of vesicular synaptobrevin and 2 plasma membrane proteins, syntaxin and SNAP-25 (11). Preventing the formation of this complex *in vivo* and *in vitro* inhibits membrane fusion and neurotransmitter release. Thus, earlier synaptic dysfunction seems to be associated with these SNARE proteins.

There is debate about how the SNARE proteins are linked to this earlier synaptic dysfunction. PrP might directly inhibit SNARE complex formation or the reduced SNARE complex is an indirect consequence of other cellular responses (12,13). Presently, we show that PrP does not affect synaptic dysfunction by directly interacting with individual SNARE proteins or the process of SNARE complex formation. SDS-resistant complex formation (Fig. 2), SNARE-driven membrane fusion (Fig. 3) and neurotransmitter release from neuronal cells (Fig. 4) were not altered in the presence of exogenously introduced PrP. Together with the recent finding of ME7 model (12), we conclude that earlier synaptic dysfunction in prion disease is not caused by direct interaction of PrP with SNARE proteins and subsequent inhibition of SNARE complex formation.

Some polyphenols are thought to be potent inhibitors of PrP^{Sc} formation and other amyloid-related diseases (39). For example, tannic acid inhibited PrP^{Sc} formation with IC_{50} of 0.1 mM (40). Epigallocatechin gallate, that is rich in green tea extracts, was also a potent inhibitor (40). They also reported that radio-labeled apigallocatechin gallate was detected in mouse brain after oral administration. In our

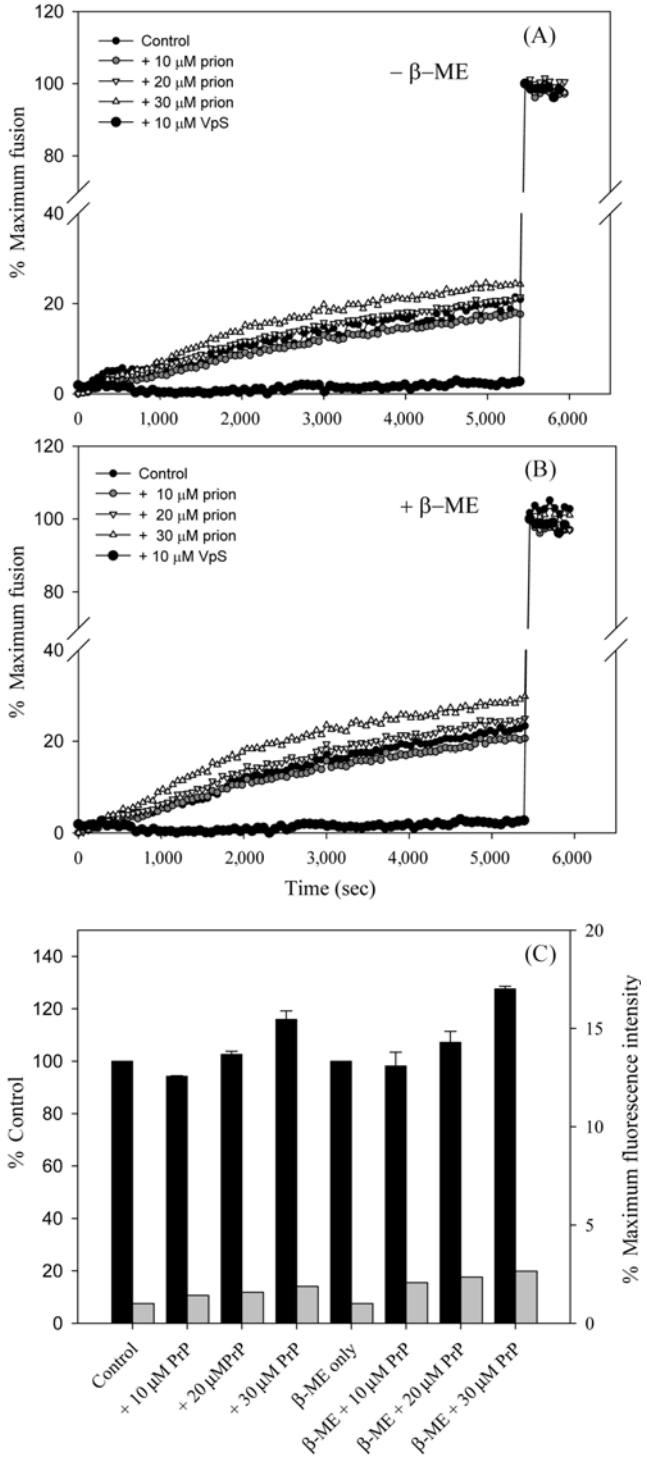

Fig. 3. Effect of PrP on SNARE-driven membrane fusion. Lipid mixing assay was performed with reconstituted SNARE-containing liposomes in the absence (A) or presence (B) of 1% β-ME. The increase of fluorescence was monitored as a function of time and the percentage of maximum fusion was obtained by dividing the fluorescence intensity at given time with maximum fluorescence intensity (MFI). (C) Gray bars indicate '% maximum fusion' measured in the absence of reconstituted SNARE proteins indicating SNARE-independent fusion ability of PrP. Black bars indicate '% control'. '% Control' was obtained by dividing % of membrane fusion measured in the presence of PrP with that measured in the absence of PrP.

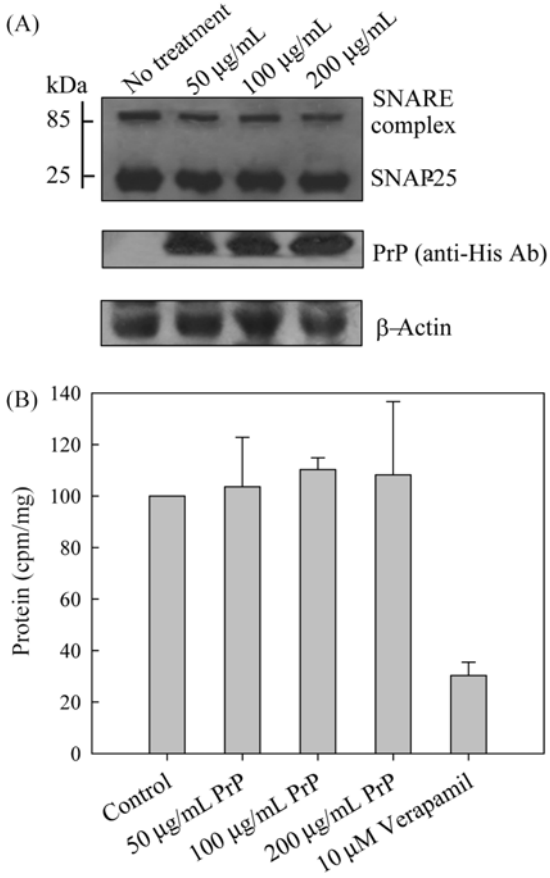

Fig. 4. Effect of transfected PrP on SNARE-driven neuroexocytosis in neuronal PC12 cells. (A) Western blot analysis for PrP transfection and SNARE complex. SNARE complex formation in PC12 cells were not inhibited by high K^+ treatment. (B) Release of noradrenalin was measured after transfection of PrP. Verapamil, an L-type calcium channel blocker, was added as a positive control.

unpublished experiments, we observed that apigallocatechin gallate did not inhibit SNARE assembly while some other polyphenols inhibited SNARE complex formation and subsequent neuroexocytosis. Thus, we assume that apigallocatechin gallate will retard PrP^{Sc} fibril formation without affecting neuroexocytosis driven by SNARE proteins.

Acknowledgments

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