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SNARE zippering is hindered by polyphenols in the neuron

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ABSTRACT

Fusion of synaptic vesicles with the presynaptic plasma membrane in the neuron is mediated by soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor (SNARE) proteins. SNARE complex formation is a zippering-like process which initiates at the N-terminus and proceeds to the C-terminal membrane-proximal region. Previously, we showed that this zippering-like process is regulated by several polyphenols, leading to the arrest of membrane fusion and the inhibition of neuroexocytosis. *In vitro* studies using purified SNARE proteins reconstituted in liposomes revealed that each polyphenol uniquely regulates SNARE zippering. However, the unique regulatory effect of each polyphenol in cells has not yet been examined. In the present study, we observed SNARE zippering in neuronal PC12 cells by measuring the fluorescence resonance energy transfer (FRET) changes of a cyan fluorescence protein (CFP) and a yellow fluorescence protein (YFP) fused to the N-termini or C-termini of SNARE proteins. We show that delphinidin and cyanidin inhibit the initial N-terminal nucleation of SNARE complex formation in a Ca^{2+}-independent manner, while myricetin inhibits Ca^{2+}-dependent transmembrane domain association of the SNARE complex in the cell. This result explains how polyphenols exhibit botulinum neurotoxin-like activity *in vivo*.

1. Introduction

The release of acetylcholine at the neuromuscular junction requires the fusion of synaptic vesicles with the presynaptic plasma membrane. This synaptic fusion event is mediated by soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor (SNARE) proteins [1–5] during the formation of the four-helical bundle termed the SNARE complex [6–8]. SNARE complex formation is a zippering-like process; initiating assembly at the N-terminal region and zipping towards the C-terminal membrane-proximal region [9–12].

We have previously shown that several polyphenols hinder neuroexocytosis by inhibiting SNARE-driven membrane fusion [13]. As a result, muscles can be paralyzed by these polyphenols [14]. Comparison of these polyphenols with a commercially available botulinum neurotoxin A (BoNT/A) (Medytox, Ochang, South Korea) in a digit abduction assay suggested that these polyphenols exhibit moderate BoNT-like activity. Results of intraperitoneal injections of these polyphenols indicated that the polyphenols are safe when they enter systemic circulation, raising the possibility that several polyphenols can function as "safe plant-derived small BoNT." Furthermore, we showed that polyphenols selectively regulate degranulation of distinct subsets of vesicles in mast cells through specific interaction with different SNARE complexes [15]. This result suggested that the formation of various SNARE complexes in cells might be under control of various polyphenols.

BoNT/A blocks neuroexocytosis by cleaving the synaptosome-associated 25 kDa protein (SNAP-25), leading to neuron-specific flaccid paralysis [16]. In contrast, the BoNT-like function of the polyphenols is elicited due to the inhibition of SNARE zippering, leading to inhibition of synaptic vesicle fusion and neurotransmitter release from the neuron. Although selected polyphenols commonly inhibited neurotransmitter release from neuronal PC12 cells, each polyphenol uniquely controlled SNARE zippering. Among the polyphenols, delphinidin and cyanidin inhibited N-terminal nucleation of SNARE zippering. Myricetin bound the middle region of the SNARE complex, stopped SNARE zippering in the

Abbreviations: SNAREs, soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptors; SNAP-25, synaptosome-associated 25 kDa protein; FRET, fluorescence resonance energy transfer.

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middle of the process, and arrested membrane fusion at the hemifusion state [13]. Because the SNARE complex is resistant to sodium dodecyl sulfate (SDS), treatment of PC12 cells with these polyphenols reduced the amount of SDS-resistant complex with differential sensitivity to pre-treated high K^+-induced depolarization.

While the regulation of SNARE zippering by the polyphenols has been extensively investigated *in vitro*, the same process in the neuron has not yet been examined [13]. Although SDS–PAGE analysis is useful for assessing the quantity of SNARE complex formed, it does not provide us with information on whether the distinctive mechanisms of zippering inhibition by each polyphenol is also utilized in the cell. Thus, in the present study, we utilized strategically placed fluorescence probes to examine how the sequential steps of SNARE assembly are uniquely regulated by each of the polyphenols.

2. Materials and methods

2.1. Construction and preparation of GFP fusion proteins

The pECFP-N1, pECFP-C1, pEYFP-N1 and pEYFP-C1 vectors were purchased from Clontech (Mountain View, CA, USA). DNA fragments encoding SNAP-25, Syntaxin1a and VAMP2 were ligated into the *Xho*I/*Kpn*I restriction enzyme sites of pECFP-C1, pECFP-N1, pEYFP-N1 and pEYFP-C1 to obtain ECFP-SNAP-25 (C-SN), Syntaxin 1a-ECFP (Stx-C), VAMP2-EYFP (Vp2-Y) and EYFP-VAMP2 (Y-Vp2).

2.2. Cell culture and transfection of PC12 cells by electroporation

PC12 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). PC12 cells were plated onto poly-D-lysine-coated culture dishes, and were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 100 μg/mL of streptomycin, 100 U/mL of penicillin, 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator. The cell cultures were split once a week, and the medium was refreshed three times a week. PC12 cells were treated with nerve growth factor (NGF) (7S, 50 ng/mL, Invitrogen) for 5 days prior to transfection.

PC12 cells were carefully rinsed with 10 mL electroporation buffer (123 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, 20 mM HEPES, pH 7.05). The cells were removed from the culture flask with two 5 mL aliquots of electroporation buffer and centrifuged at 1000×*g* for 5 min. The supernatant was discarded and the cell pellet was resuspended in 1 mL electroporation buffer. For transfection, the cell suspension was mixed with plasmid DNA in a 4-mm electroporation cuvette. After incubation for 2–5 min on ice, electroporation was performed using the following parameters: 500 μF, 220 V, ∞ Ω (Gene Pulser, Bio-Rad). The transfected cells were thoroughly resuspended in serum containing medium and incubated at 37 °C, 5% CO_2 for 24 h.

2.3. Measurement of SNARE complex formation in PC12 cells by spectrophotometry

PC12 cells were transfected with pairs of fluorescence resonance energy transfer (FRET) plasmid DNA engineered to express C-SN, Stx-C, Y-Vp2, Vp2-Y and Y-Vp2. The cells were depolarized with high K^+ buffer (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). The fluorescence intensity was monitored in two channels with an excitation wavelength of 435 nm and emission wavelengths of 480 and 530 nm. The final emission data were corrected for background fluorescence, and normalized with respect to its initial intensity. The fluorescence intensity was measured using a Synergy H1 Hybrid microplate reader (Biotek Instruments) using the bottom read mode.

2.4. Confocal microscopy and fluorescence recovery after bleaching assay

PC12 cells were transfected with plasmid DNAs expressing fusion proteins. PC12 cells cultured on glass coverslips were transfected with constructs expressing cyan fluorescent protein (CFP)- and yellow fluorescent protein (YFP)-tagged proteins and fixed 24 h post-transfection with 3.7% paraformaldehyde for 10 min. For detection of CFP, cells were viewed with a fluorescence microscope (Zeiss LSM510 Meta confocal microscope, Jena, Germany) using a filter set with an excitation filter of 405 nm and detected at 465–510 nm. YFP-expressing cells were excited with the 514-nm line of the argon laser, and detected at 520–555 nm. Images were captured with a cooled charge-coupled device (CCD) camera (Quantix 57, Photometrics, Tucson, AZ, USA). Several regions of interest (ROI) per cell were photobleached in the YFP channel, using the 514-nm argon laser line at 100% intensity. Bleaching experiments were performed using the fluorescence recovery after photobleaching (FRAP)-wizard of the Zeiss Confocal Software Version 2.5 Build 1347 (Leica Microsystems, Mannheim, Germany). A laser-scanning confocal microscopic photobleaching technique was used to document that FRET occurred by showing that the intensity of the donor CFP fluorescence increased after its acceptor YFP was photobleached. CFP images were collected before and after photobleaching to measure changes in donor fluorescence. FRET efficiency was expressed as the ratio of CFP fluorescence gain after YFP photobleaching. To calculate the apparent FRET efficiency in the ROI, the Leica software uses the formula FRET = [(EDpost − EDpre)/EDpost] × 100, where FRET represents the efficiency and ED represents the emitted donor fluorescence before (EDpre) and after (EDpost) photobleaching of the acceptor.

3. Results

3.1. Monitoring SNARE assembly in PC12 cells using a FRET-based assay

SNARE zippering starts at the N-termini of SNARE motifs and proceeds to the C-termini [17,18]. The term 'zippering' is used because 4 helix bundle formation is a directional and gradual process. Furthermore, the structure of the *cis* SNARE complex, where 2 transmembrane domains (TMD) of the complex are sitting on the same membrane, shows that α-helical SNARE complexes extend throughout the transmembrane domains. Thus, we designed our experiment such that the initial N-terminal nucleation of the SNARE complex could be probed by an N-termini-tagged FRET pair ($FRET_N$), and the engagement of the TMDs could be probed by a C-termini-tagged FRET pair ($FRET_C$). For this purpose, two sets of FRET pairs were prepared by fusing SNARE proteins with CFP and YFP (Fig. 1). For $FRET_N$, CFP was placed at the N-terminus of SNAP-25 (C-SN) and YFP was N-terminally fused to VAMP2 (Y-Vp2). WT Syntaxin1 was used when $FRET_N$ was measured. For $FRET_C$ measurement, untagged WT SNAP-25 was used while CFP and YFP were placed at the C-termini of the transmembrane domains of Syntaxin1 (Stx-C) and VAMP2 (Vp2-Y), respectively.

After transfecting PC12 cells with the plasmids coding for the CFP-YFP FRET pairs, expression of the fusion proteins was confirmed by Western blot (Fig. 2A). The cells used for measurement of $FRET_N$ expressed the 18 kDa WT VAMP2, 25 kDa WT SNAP-25, 45 kDa Y-Vp2 and 52 kDa C-SN proteins. Cells for $FRET_C$ measurement expressed 18 kDa WT VAMP2, 25 kDa WT SNAP-25, 45 kDa

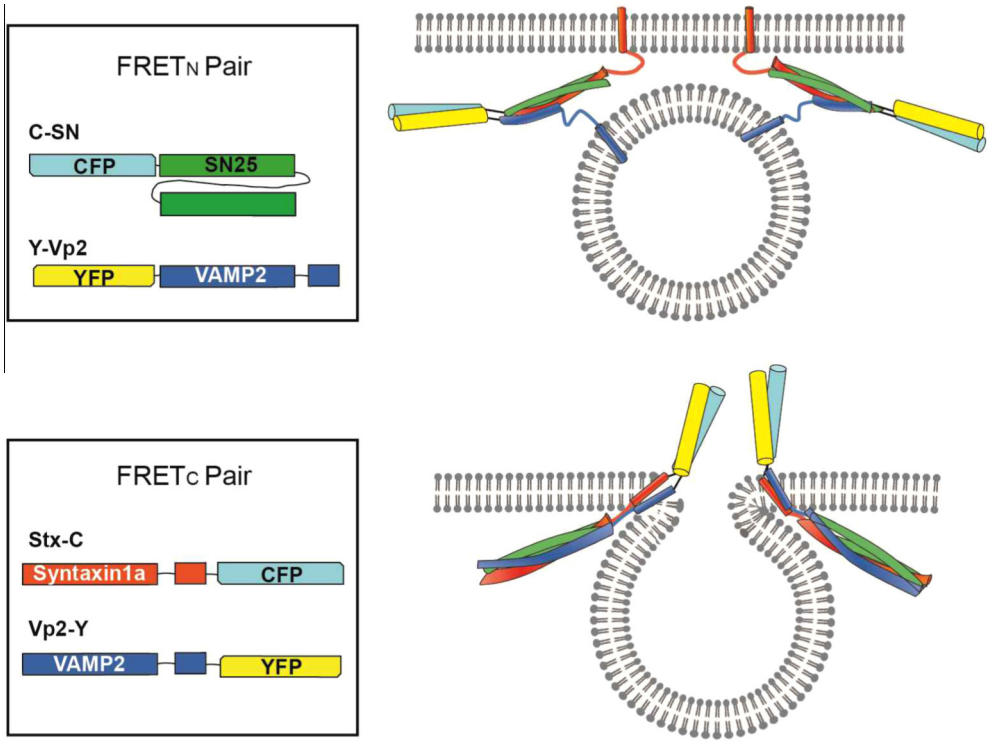

Fig. 1. Domain structure of FRET pairs. CFP-SNAP25(C-SN), YFP-VAMP2(Y-Vp2), Syntaxin 1a-CFP (Stx-C), and VAMP2-YFP(Vp2-Y). Schematic models of SNARE complex assembly from N-terminal zippering to TMD interaction based on FRET pairs.

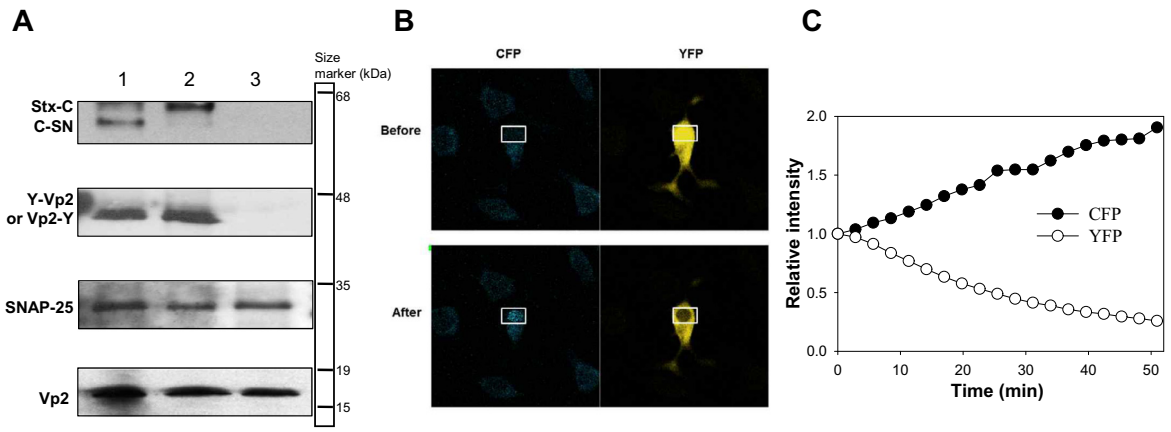

Fig. 2. FRET of green fluorescent protein-tagged SNARE proteins in PC12 cells. (A) Co-immunoprecipitation of CFP-SNAP25 (C-SN), Syntaxin-CFP (Stx-C), YFP-VAMP2 (Y-Vp2) and VAMP2-YFP (Vp2-Y) in PC12 cells using anti-VAMP2 antibodies. The immunoprecipitated proteins were blotted against mouse anti-SNAP-25, rabbit anti-VAMP2, and mouse anti-syntaxin antibodies. Lane 1, cells expressing $FRET_N$ pairs (C-SN and Y-Vp2); lane 2, cells expressing $FRET_C$ pairs (Stx-C and Vp2-Y); and lane 3, control PC12 cells without transfection. Molecular mass markers are 68, 48, 35, 19, and 15 kDa. (B) A set of mixed Stx-C and Vp2-Y images of PC12 cells taken before and after acceptor photobleaching. The region of photobleaching is indicated by the white outlined box. The intensity of the donor Stx-C emission in PC12 cells increased after photobleaching of the acceptor Vp2-Y, while the acceptor Vp2-Y signal decreased. Photobleaching of YFP increased CFP fluorescence, indicating that the emitted light from C-SN was well transferred to Y-Vp2 by SNARE complex formation before photobleaching. (C) Fluorescence intensity at a given time was normalized by the initial intensity.

Vp2-Y and 60 kDa Stx-C proteins. Complex formation between the CFP- and YFP-fused SNARE proteins was then examined by measuring FRET between the pair. Photobleaching of the acceptor YFP led to recovery of the donor CFP signal, indicating that FRET occurred between the pairs in PC12 cells (Fig. 2B and C).

Then, we measured FRET changes for $FRET_N$ and $FRET_C$ individually after high K^+ pretreatment. A high concentration of KCl (50 mM) is known to induce depolarization of PC12 cells, leading to SNARE-mediated membrane fusion and release of neurotransmitters [19]. Thus, pretreatment of PC12 cells with 50 mM KCl is thought to induce SNARE recycling. Treatment with high K^+ induced a rapid rise in both $FRET_N$ and $FRET_C$, followed by a fast decrease (Fig. 3A), which could be observed in both the pretreatment and main treatment steps. These observations are in excellent agreement with previous reports, which showed that four-helical bundle formation is also tightly coupled to the TMD [20–23]. We interpret this as an indication of *cis* complex formation of entire SNAREs and subsequent recycling of complexes, respectively. We then replaced high K^+ with low K^+ (5 mM) when the FRET values were at their minima and monitored the changes in FRET values as a function of time. FRET values increased rapidly indicating that the SNARE complex was forming, presumably due

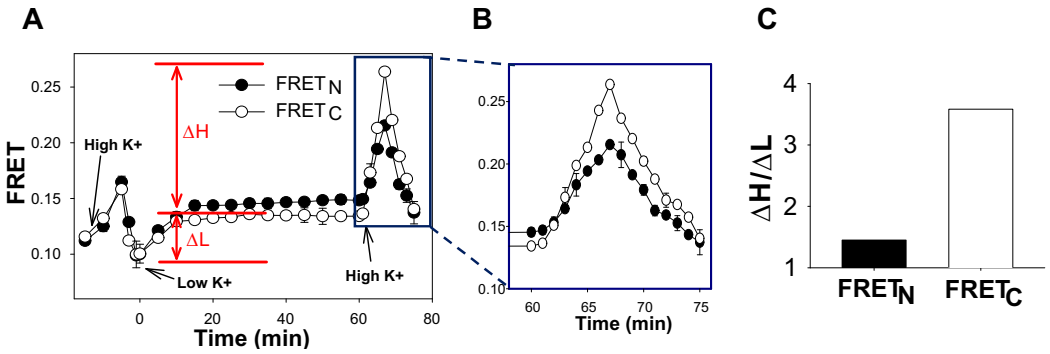

Fig. 3. FRET changes $FRET_N$ and $FRET_C$ after high K^+ pretreatment. (A) FRET between C-SN and Y-Vp2 ($FRET_N$) was measured after high K^+ pretreatment. After changing to normal growth media ($t = 0$, Low K^+ solution) FRET was measured for 60 min, followed by repeat treatment with the high K^+ solution. FRET between Stx-C and Vp2-C ($FRET_C$) was measured in the same manner. The boxed area is magnified in (B). (B) Enlarged view of FRET changes after high K^+ treatment. The changes in FRET values in the low K^+ state and high K^+ state are termed ΔL and ΔH, respectively. (C) Dependence of N-terminal nucleation of SNARE complex formation ($FRET_N$) and TMD interaction ($FRET_C$) on the calcium trigger.

to the reloading of the readily releasable pool (RRP). FRET values reached a plateau in ~10 min and did not change until the cells are treated again with high K^+. This second high K^+ treatment induced the same sudden increase and subsequent decrease of FRET values as observed with the first high K^+ treatment. An enlarged view of the data after high K^+ treatment (boxed in Fig. 3B) shows that both $FRET_N$ and $FRET_C$ values reach their maxima ~5 min after treatment, which is the same time course for neurotransmitter release from PC12 cells [19].

Intriguingly, $FRET_N$ and $FRET_C$ showed different degrees of FRET change depending on conditions. The rise of $FRET_C$ was ~30% smaller than that of $FRET_N$ upon changing to the low K^+ conditions (ΔL of $FRET_N = 1.3 * \Delta L$ of $FRET_C$, where ΔL is FRET increase at low K^+ state). In contrast, the escalation induced by high K^+ is much greater for $FRET_C$ than for $FRET_N$ (ΔH of $FRET_N = 0.53 * \Delta H$ of $FRET_C$, where ΔH is FRET increase at high K^+ state). We expect that FRET changes (ΔL) measured at the low K^+ state represents Ca^{2+}-independent SNARE assembly, whereas Ca^{2+}-dependent complex formation could be calculated by FRET changes (ΔH) after depolarization with the high K^+ solution. This observation suggests that the association of TMDs is much more expedited by calcium while the N-terminal nucleation of the SNARE folding process is less affected by calcium (Fig. 3C).

3.2. Monitoring inhibition of SNARE assembly by polyphenols in PC12 cells using a FRET-based assay

Previously, it was shown that myricetin stopped SNARE zippering in the middle of membrane fusion resulting in a half-zipped replication fork-like structure, while delphinidin and cyanidin inhibited the N-terminal nucleation of SNARE zippering [13]. This unique regulatory effect of each polyphenol on SNARE zippering was examined by measuring changes in $FRET_N$ and $FRET_C$.

First, the effect of each inhibitory polyphenol (10 μM) on $FRET_N$ and $FRET_C$ was measured upon low K^+ treatment. Delphinidin, cyanidin and myricetin inhibited the ΔL of $FRET_N$ by ~40%, indicative of reduced N-terminal nucleation (Fig. 4A). Similarly, the ΔL of $FRET_C$ values was also decreased by these polyphenols (Fig. 4B). Kaempferol, a control polyphenol, did not affect either $FRET_N$ or $FRET_C$. The relative ratios of the ΔL for both $FRET_N$ and $FRET_C$ were almost identical (Fig. 4C). This result indicates that the reduction in the TMD interaction in the absence of calcium is a natural consequence of reduced N-terminal nucleation.

Upon high K^+ treatment, the ΔH of $FRET_N$ and $FRET_C$ were also inhibited by the 3 inhibitory polyphenols (Fig. 4B and D). However, the degree of inhibition was quite different depending on the polyphenols added. The ratio of reduction in ΔH and ΔL of $FRET_N$ was similar among the 3 inhibitors (Fig. 4D), suggesting that inhibition of N-terminal nucleation is not affected by the presence of calcium. Because N-terminal nucleation should precede transmembrane domain association, $FRET_N$ reduction naturally accompanies $FRET_C$ reduction. Delphinidin and cyanidin reduced $FRET_C$ in proportion to the change of $FRET_N$, which is consistent with our *in vitro* result that these two polyphenols inhibit only the N-terminal nucleation step of SNARE complex formation. In contrast, myricetin had an additional inhibitory effect on $FRET_C$. We observed only a 24% increase in $FRET_C$ in the presence of myricetin, which corresponds to a 76% reduction in calcium-triggered TMD interactions. This difference between $FRET_N$ and $FRET_C$ values as a result of myricetin treatment suggests that the N-terminally zipped partial complex did not lead to a TMD association upon triggering by calcium. This result is consistent with the results of our membrane fusion assay performed using reconstituted vesicles, where myricetin had binding sites around the 0 layer, and inhibited the progression of zippering of the N-terminally nucleated partial complex [13].

4. Discussion

We have previously shown that several polyphenols intercalate into the inner layer of the SNARE complex, and that the binding of polyphenol to SNARE proteins undergoing the zippering process inhibits membrane fusion. Among the SNARE-inhibitory polyphenols, delphinidin and cyanidin had binding sites at the N-terminal region of the SNARE complex's four-helical bundle, and inhibited the nucleation of SNARE zippering [13]. This early stage interference inhibited docking of vesicles. In contrast, myricetin had 1 weak binding site at the N-terminal region and 1 strong binding site near the 0 layer of the SNARE complex. Binding of myricetin near the 0 layer during SNARE zippering resulted in the arrest of zippering midway through the process and membrane fusion stopped at the hemifusion state. Thus, in the present study, the effects of these 3 polyphenols, which were analyzed previously using purified SNARE proteins and reconstituted vesicles, on neuronal SNARE assembly were confirmed in PC12 cells. By measuring FRET changes between CFP- or YFP-fused SNARE proteins we verified that each polyphenol indeed inhibited SNARE assembly in the same manner that we observed *in vitro*.

Relative ΔL values for $FRET_N$ and $FRET_C$ were similarly inhibited by polyphenols (Fig. 4), indicating that reduced N-terminal nucleation accompanies a similar degree of reduction in the TMD interactions when calcium is not present. Furthermore, the relative ΔH

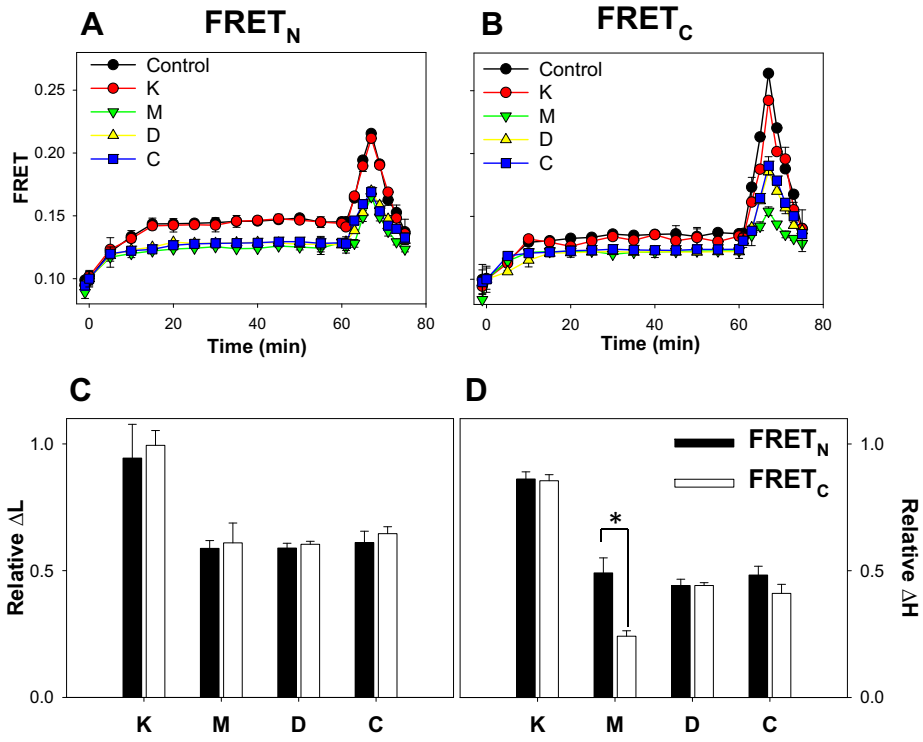

Fig. 4. Inhibitory effect of polyphenols on SNARE assembly. (A and B) The changes of $FRET_N$ and $FRET_C$ as a function of time in the presence of 10 μM kaempferol (K), myricetin (M), delphinidin (D) and cyanidin (C). (C) Relative inhibitory effects of polyphenols on ΔL for $FRET_N$ and $FRET_C$. (D) Relative inhibitory effects of polyphenols on ΔH for $FRET_N$ and $FRET_C$.

values of $FRET_N$ and $FRET_C$ were very similar except for myricetin. Myricetin reduced the calcium-triggered TMD interaction by as much as ~76% compared to the control (Fig. 4D). This result suggests that further progression of SNARE zippering of the N-terminally zipped partial complex is still inhibited even in the presence of a calcium trigger.

In summary, calcium triggers C-terminal SNARE assembly through the TMD and leads to neurotransmitter release (Fig. 3). Myricetin not only inhibits N-terminal nucleation of SNARE zippering, but also prevents the calcium-triggered TMD interaction, which leads to inhibition of neurotransmitter release. In contrast, delphinidin and cyanidin inhibited only the N-terminal nucleation of SNARE. In conclusion, the unique inhibition patterns of each polyphenol on SNARE zippering that were analyzed *in vitro* using purified SNARE proteins and reconstituted vesicles were also observed in neuronal PC12 cells.

Acknowledgments

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