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財団法人　日中医学協会　御中

貴財団より助成金を受領して行った研究テーマについて報告いたします。

添付資料：**研究報告書**

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２．研究テーマ

新規分子 PRIP の開口分泌における役割の解明

３．成果の概要

SNAP-25 (synaptosomal-associated protein 25) is a member of SNARE complex which is the minimal machinery for membrane fusion, the final step of regulated exocytosis. Phosphorylated SNAP-25 was dephosphorylated by protein phosphatase-1, whose activity was regulated by PRIP, thus regulating exocytosis. The results provide the first information regarding the phosphatases responsible for phospho-modulation of SNAP-25 and the regulation of exocytosis.

４．研究業績

（１）学会における発表　　無・(有)（学会名・演題）

1. Roles of PRIP in phospho-regulation of exocytosis through the interaction with protein phosphatases. The 7th Korea-Japan Conference on Cellular Signaling for Young Scientist, Ulsan, Korea, Feb 17-18, 2012.
2. Phospho-dependent modulation of exocytosis by PRIP. The 10th JBS Biofrontier Symposium on New Aspects of Phospholipid Biology and Medicine, Fukuoka, Japan, Nov 14-16, 2011.
3. Involvement of PRIP in exocytosis through phospho-dependent regulation of SNAP-25. 第８４回日本生化学会大会、京都市、2011.9.

（２）発表した論文　　無・(有)（雑誌名・題名）

Gao, J., Takeuchi, H., Zhang, Z., Fukuda, M. and Hirata, M.: Phospholipase C-related but catalytically inactive protein (PRIP) modulates synaptosomal-associated protein 25 (SNAP-25) phosphorylation and exocytosis. J Biol Chem. 287:10565-10578, 2012

５．**指導責任者の意見**（指導責任者がご記入・ご捺印ください）

中国・河北医科大学を卒業後、来日し本学大学院博士課程を優秀な成績で終了した高靖は現在、当教室で助教として教育および研究に従事しています。幸運にも貴財団からの援助を得て、彼女は本研究を遂行することができました。学部学生教育や大学院生の実験指導も行いながら、着実に研究成果を重ねて学会発表のみならず原著論文を作製するまでなし得たのは彼女の弛まぬ努力と優れた才能の賜であると言えます。将来中国に帰国後も必ず中国における医学研究に貢献してくれるものと信じています。

指導責任者署名 平田雅人 ㊞

—日中医学協会助成事業—

新規分子 PRIP の開口分泌における役割の解明

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要旨:
Exocytosis is one of the most fundamental cellular events. The basic mechanism of the final step, membrane fusion, is mediated by the formation of the SNARE complex, which is modulated by the phosphorylation of proteins controlled by the concerted actions of protein kinases and phosphatases. We have previously shown that a protein phosphatase-1 (PP1) anchoring protein, PRIP (phospholipase C-related, but catalytically inactive protein), has an inhibitory role in regulated exocytosis. The current study investigated the involvement of PRIP in the phospho-dependent modulation of exocytosis. Dephosphorylation of SNAP-25 (synaptosome-associated protein of 25kDa) was mainly catalyzed by PP1 and the process was modulated by wild-type PRIP, but not by the mutant (F97A) lacking PP1-binding ability in *in vitro* studies. We then examined the role of PRIP in phospho-dependent regulation of exocytosis in cell-based studies using a pheochromocytoma cell line, PC12 cells, that secrete noradrenalin. Exogenous expression of PRIP accelerated the dephosphorylation process of phosphorylated SNAP-25 after forskolin or phorbol ester treatment of the cells. The phospho-states of SNAP-25 were correlated with noradrenalin secretion, which was enhanced by forskolin or phorbol ester treatment and modulated by PRIP expression in PC12 cells. Both SNAP-25 and PP1 were co-precipitated in anti-PRIP immunocomplex isolated from PC12 cells expressing PRIP. Collectively, together with our previous observation regarding the roles of PRIP in PP1 regulation, these results suggest that PRIP is involved in the regulation of the phospho-states of SNAP-25 by modulating the activity of PP1, thus regulating exocytosis.

Key words:
cAMP-dependent protein kinase, exocytosis, phospholipase C, protein phosphatase, SNARE

緒言:
Protein phosphorylation and dephosphorylation through activation of protein kinases and phosphatases play an important role in the regulation of exocytosis. Fewer studies regarding the phosphatases responsible for the phospho-regulation of exocytosis have been performed than those regarding kinases. Furthermore, the combination of specific substrate proteins implicated in exocytosis, specific kinase and phosphatase, and their regulation to modulate exocytosis are still unknown.
Phospholipase C-related, but catalytically inactive protein (PRIP) was originally identified in this laboratory as a novel D-*myo*-inositol 1, 4, 5-trisphosphate [Ins(1,4,5)P_3] binding protein, whose name was derived from the lack of catalytic activity in spite of the similarity to phospholipase Cδ-1 (1-6). Further studies revealed that PRIP has a number of binding partners, including the catalytic subunit of protein phosphatase 1α (PP1α) and PP2A (7, 8), phosphorylated (active) form of Akt (9). Thus, PRIP is an unique molecule which associates with both multiple phosphatases and a kinase, suggesting that PRIP participates in the phosphoregulation of cellular events, by recruiting these enzymes to where the event occurs if PRIP can approach. We have recently reported that exocytosis of various peptide hormones such as gonadotropins and insulin was up-regulated in PRIP knock-out mice (10, 11), indicating that PRIP is likely to be involved in dense-core vesicle exocytosis in a negative manner. The molecular mechanisms underlying the inhibition of exocytosis by PRIP are currently being studied in the laboratory. In the present study, we investigated the possible involvement of PRIP in the phospho-regulation of exocytosis through modulation of the dynamics of protein phosphorylation.

研究方法:
Noradrenalin Secretion Assay: PC12 cells were labeled with [^{3}H]noradrenalin (NA). The secretion of

[^{3}H]NA was triggered with high-K^{+}/PSS (81 mM NaCl, 70 mM KCl). The radioactivity of [^{3}H]NA remaining in cells and secreted into the medium was measured by a liquid scintillation counter.

In vitro Phosphorylation and Dephosphorylation of SNAP-25: GST-tagged SNAP-25 was phosphorylated with [γ-^{32}P]ATP using the catalytic subunit of PKA. The mixture was separated by SDS-PAGE, followed by CBB staining and autoradiography. For the dephosphorylation assay, GST-tagged SNAP-25 immobilized on glutathione beads was phosphorylated as described above, followed by de-phosphorylation by PP1, PP2A, or PP2B in an appropriate buffer solution. The radioactivity of released ^{32}P was counted using a liquid scintillation counter, and beads were analyzed by SDS-PAGE for CBB staining and autoradiography.

Labeling of PC12 Cells and Immunoprecipitation for Phosphorylation Assay: PC12 cells were labeled with [^{32}P]orthophosphate. After treating the cells with the substance of interest, cells were lysed and the cell extract were subjected to immuonoprecipitation by anti-SNAP-25 antibody and the precipitates were examined by SDS-PAGE for autoradiography.

結果：

Regulation of exocytosis by protein phosphorylation: We previously found that the absence of ATP after permeabilization of PC12 cells diminished Ca^{2+}-triggered exocytosis (12), and this diminishment is assumed to be caused by the conversion of membrane PtdIns(4,5)P_2 to phosphatidylinositol phosphate and then phosphatidylinositol in the absence of ATP. We assumed that protein phosphorylation involved in exocytosis is also implicated in its regulation and it was confirmed using a protein phosphatase inhibitor, calyculin A, which partially rescued the diminishment by about 25 % (Data not shown). PtdIns(4,5)P_2 was not increased by calyculin A, although permeabilization decreased PtdIns(4,5)P_2 and incubation in the presence of ATP increased PtdIns(4,5)P_2 (Data not shown). The results clearly indicate that protein phosphorylation is involved in the regulation of exocytosis in a positive manner.

Dephosphorylation of SNAP-25: Many proteins important for exocytosis can be phosphorylated by various protein kinases. One of the most investigated SNARE proteins is SNAP-25, the phosphorylation of which has been previously reported to be catalyzed by both PKA and PKC to enhance exocytosis (13-16). Thus, we focused on the phospho-modulation of exocytosis *via* SNAP-25.

GST-fused SNAP-25 was phosphorylated by the catalytic subunit of PKA using [γ-^{32}P]ATP (Fig. 1A), which was subjected to measuring phosphatase activity of the catalytic subunit of PP1, PP2A or PP2B. PP1 caused the release of ^{32}P from GST-SNAP-25 in a dose-dependent manner, and PP2A also catalyzed the release but to a lesser extent, whereas PP2B showed no activity (Fig. 1B), indicating that SNAP-25 phosphorylated by PKA was mainly dephosphorylated by PP1 and to a lesser extent by PP2A *in vitro*.

We have previously shown that PRIP is a negative modulator of PP1 (7). PRIP binds to PP1 to inhibit the phosphatase activity. When PRIP-1 itself is phosphorylated by PKA at residue T94, PP1 could no longer associate with PRIP to be an active form. Thus, we examined the effect of PRIP on dephosphorylation of SNAP-25 by PP1. The release of ^{32}P from phosphorylated SNAP-25 was inhibited by the wild-type PRIP-1, but not by the mutant PRIP-1, whose residue Phe97 was replaced with Ala, lacking PP1 binding ability (7) (Fig. 1C). Dephosphorylation of SNAP-25 catalyzed by PP1 was not inhibited by previously phosphorylated PRIP-1 (Fig. 1D). The results indicate that PRIP could be involved in the modulation of the phospho-state of SNAP-25 through regulating the activity of PP1.

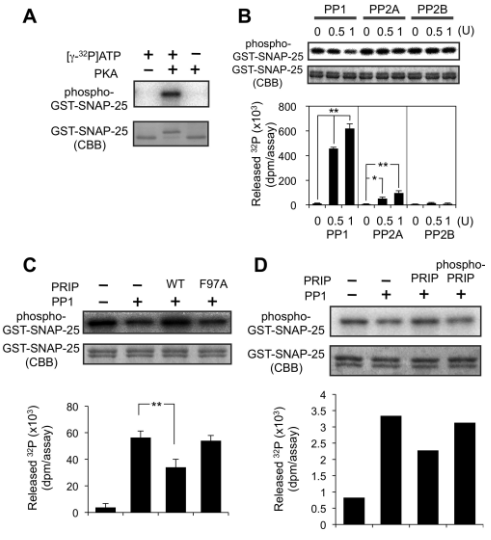
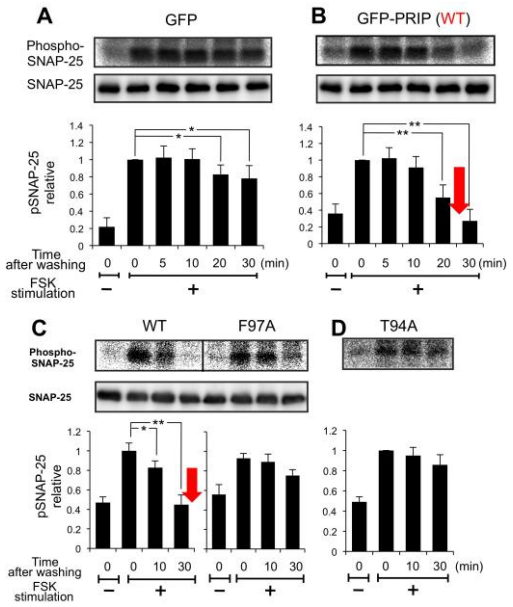


FIGURE 1. Dephosphorylation of SNAP-25 was mainly catalyzed by PP1 and modulated by PRIP-1 in vitro. (A) GST-tagged SNAP-25 was phosphorylated with [γ-^{32}P]ATP using the catalytic subunit of PKA. (B) Phosphorylated GST-tagged SNAP-25 was de-phosphorylated by PP1, PP2A, or PP2B. (C) Recombinant PRIP-1 (WT) or the mutant F97A was included in the phosphatase reaction mixture. (D) PRIP-1 was phosphorylated in advance by non-radioactive ATP plus the catalytic subunit of PKA (pPRIP), followed by a dephosphorylation assay of SNAP-25.

Regulation of dephosphorylation of SNAP-25 by PRIP-1: We then examined the roles of PRIP in regulating the phospho-state of SNAP-25 in PC12 cells labeled with [^{32}P]orthophosphate. Forskolin treatment caused robust ^{32}P incorporation into SNAP-25, slowly decreasing for up to 30 min after the removal of forskolin (Fig. 2A). However, expression of wild type PRIP-1(Fig. 2B), but not the mutant PRIP-1 (F97A; Fig. 2C), which lacks PP1 binding ability, or PRIP-1 (T94A; Fig. 2D), which is not phosphorylated and therefore keeps PP1 sequestered and inactivated, promoted the dephosphorylation process of SNAP-25. These results indicate that the dephosphorylation process of SNAP-25 catalyzed mainly by PP1 was accelerated by the presence of PRIP-1 in PC12 cells, and PP1 binding ability is required for PRIP to execute the role in regulating the dephosphorylation of SNAP-25.

FIGURE 2. Dephosphorylation of SNAP-25 was modulated in PC12 cells expressing PRIP-1. PC12 cells expressing GFP (A), GFP-PRIP-1 (WT) (B), GFP-RPIP-1 (F97A) (C) or GFP-PRIP-1 (T94A) (D) were labeled with [^{32}P]orthophosphate, followed by stimulation with 50 μM FSK for 5 min. After removing the stimulus, cells were left for the time period indicated. Phosphorylation of SNAP-25 was analyzed by immunoprecipitation followed by autoradiography and Western blotting.



Noradrenalin secretion induced by high-K^+ from PC12 cells, which were treated similarly to the description in Figure 2, was also examined. As shown in Figure 3, the results indicate that PRIP-1 is involved in the regulation of NA secretion through modulating the dephosphorylation of some proteins important for exocytosis. This correlated with the regulation of the phospho-state of SNAP-25 by PRIP-1 through PP1 binding.

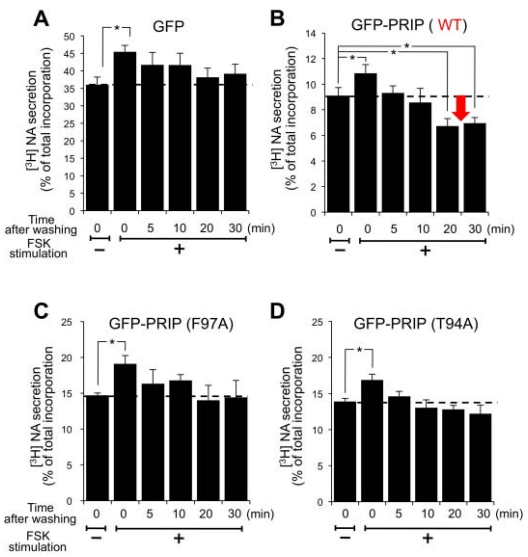


FIGURE 3. [^{3}H]NA secretion was modulated by PRIP-1. PC12 cells expressing GFP (A), GFP-PRIP-1 (WT) (B), GFP-RPIP-1 (F97A) (C) or GFP-PRIP-1 (T94A) (D) were labeled with [^{3}H]NA, followed by stimulation with 50 μM FSK for 5 min. After removing the stimulus, cells were left at room temperature for 5, 10, 20 or 30 min, followed by [^{3}H]NA secretion assay with high-K^+ solution for 5 min.

Complex formation of PRIP-1 with PP1 and SNAP-25 in PC12 cells: SNAP-25 is mainly localized at the plasma membrane, while PP1 exists throughout the cytosol in the cells, therefore PP1 needs to be recruited to the site where SNAP-25 is localized in order to function in exocytosis. To examine if PRIP helps PP1 to be recruited to the site where SNAP-25 is localized and exocytosis takes place, we performed a co-immunoprecipitation assay using PC12 cells expressing PRIP-1. As shown in the figure, SNAP-25 formed complex with PRIP-1, PP1 and syntaxin (Fig. 4A); however, PP1 was only co-precipitated with WT of PRIP-1, not with F97A (Fig. 4B). PC12 cells expressing either WT or T94A mutant of PRIP-1 were first treated with forskolin for 5 min to induce the phosphorylation of PRIP-1 probably along with SNAP-25, followed by immunoprecipitation by anti-PRIP-1 antibody. As shown in Figure 4C, forskolin treatment reduced the amount of PP1 immunoprecipitated with PRIP-1 from PC12 cells expressing WT, but not cells expressing T94A of PRIP-1, despite a similar amount of SNAP-25. These results support our

assumption that PRIP-1 recruits PP1 to SNAP-25 by the complex formation, probably along with syntaxin.

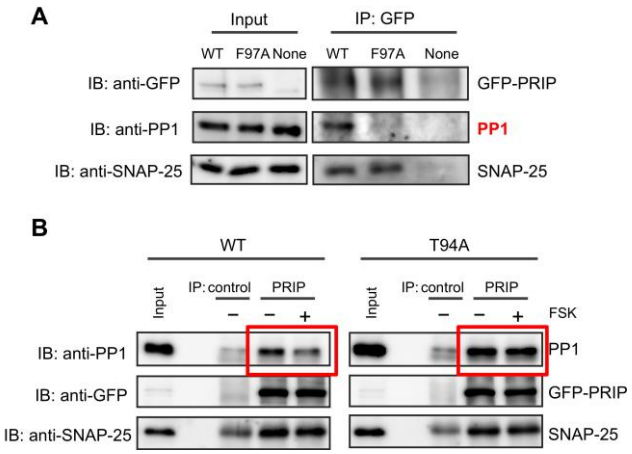


FIGURE 4. Interaction of PRIP-1, SNAP-25 and PP1 in PC12 cells. (A) PC12 cells expressing GFP alone (none) or GFP-PRIP-1 (WT) were subjected to co-immunoprecipitation assay using anti-SNAP25 antibody. (B) PC12 cells expressing GFP (none), GFP-PRIP-1 (WT) or GFP-PRIP-1 (F97A) were subjected to co-immunoprecipitation assay with anti-GFP antibody using an Seize X protein A Immunoprecipitation Kit. (C) PC12 cells expressing GFP-PRIP-1 (WT) or GFP-PRIP-1 (T94A) were first stimulated to phosphorylate PRIP-1 itself with FSK (50 μM) for 5 min, followed by immunoprecipitation by anti-PRIP-1 antibody (PRIP). The immunoprecipitates were analyzed by Western blotting.

考察：

Collecting the data presented in this study, a model explaining that PRIP modulates the phospho-states of SNAP-25 and exocytosis is shown in Figure 5. PRIP recruits PP1 and PP2A to the site where t-SNARE proteins exist, but inhibits PP1 activity, probably with the aid of the PH and C2 domains (Fig. 5A). When the intracellular cAMP level is elevated or PKC is activated by cellular stimulation, PKA and PKC phosphorylate both SNAP-25 and PRIP. Following the phosphorylation of PRIP, PP1 is released to be active near SNAP-25; thus, PP1 can dephosphorylate SNAP-25 effectively to abolish the effect (Fig. 5B).

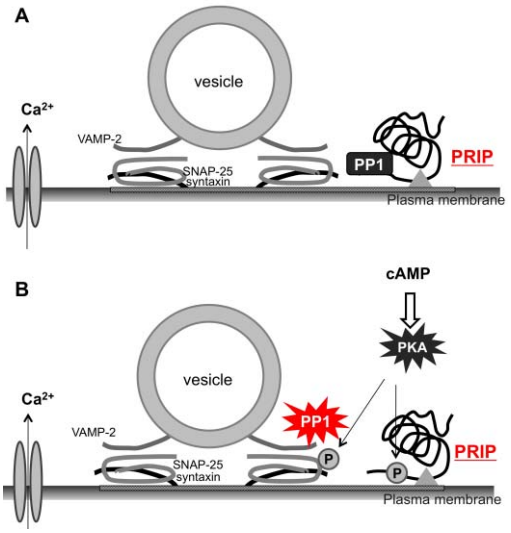


FIGURE 5. Schematic representation of the role of PRIP in phospho-dependent regulation of SNAP-25. (A) Basal condition, and (B) PKA or PKC activated condition (see DISCUSSION).

The current study showed the possible involvement of PRIP in PKA and PKC-dependent phospho-modulation of regulatory exocytosis by regulating the location and activities of protein phosphatases, PP1 and PP2A, using PC12 cells expressing PRIP-1. To our knowledge, there have been few studies regarding the de-phosphorylation (OFF) process of SNARE proteins for exocytosis compared to those regarding the phosphorylation (ON) process. For the first time, we here elucidated that PP1 is a major phosphatase responsible for the OFF process, which is regulated by PRIP. Further studies are clearly required using cells intrinsically expressing PRIP for a more physiological point of view. Other issues to be addressed are the role of PP2A binding of PRIP and the regulation of catalytic activity in phospho-dependent modulation of exocytosis, although the participation of PP2A appears to be reduced. Furthermore, other proteins of exocytosis, including syntaxin as a substrate, have been reported to participate in the phospho-dependent regulation of exocytosis (17,18). Whether PRIP modulates the phospho-states of these proteins should also be investigated to better understand the mechanism of the OFF process in the phospho-modulation of exocytosis.

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