J. Biochem. **136**, 245–253 (2004)
DOI: 10.1093/jb/mvh116

Effect of Forskolin on Synaptotagmin IV Protein Trafficking in PC12 Cells

Mitsunori Fukuda[1,*] and Akitsugu Yamamoto[2]

[1]Fukuda Initiative Research Unit, RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama 351-0198; and [2]Nagahama Institute of Bio-Science and Technology, Nagahama, Shiga 526-0829

Received May 31, 2004; accepted June 7, 2004

Synaptotagmin IV (Syt IV) was originally described as an immediate early gene product induced by forskolin or membrane depolarization in PC12 cells; however, nothing is known about the subcellular localization and transport of the newly translated Syt IV protein in PC12 cells. In this study, we investigated the transport mechanism of Syt IV protein induced by forskolin and found that forskolin treatment dramatically increases the Syt IV protein level (approximately 10-fold, to a level comparable to that of Syt IX) and promotes the transport of Syt IV protein from the Golgi to the cell periphery by a microtubule-dependent motor(s). The expression levels and subcellular localizations of two major Syt isoforms (I and IX) in PC12 cells, on the other hand, were unaffected by such treatment. Immunoelectron microscopic analysis showed that some Syt IV signals are clearly associated with dense-core vesicles in forskolin-treated PC12 cells, although the majority of the Syt IV molecules at the cell periphery were present on clear vesicular structures other than dense-core vesicles. An N-terminal antibody-uptake experiment indicated that Syt IV–containing vesicles in forskolin-treated PC12 cells undergo Ca^{2+}-dependent exocytosis, because uptake of the anti-Syt IV-N antibody from the culture medium was slightly, but significantly, increased after forskolin treatment. Our results indicate that forskolin (or the increased cAMP level) is important for the transport of the Syt IV protein from the Golgi to the cell periphery, but not sufficient for the sorting of all Syt IV molecules to mature dense-core vesicles.

Key words: dense-core vesicle, forskolin, Golgi, immediate early gene, regulated exocytosis, synaptotagmin.

Abbreviations: BFA, brefeldin A; CREB, cAMP-responsive element-binding protein; ER, endoplasmic reticulum; LTP, long-term potentiation; NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Syt(s), synaptotagmin(s); TGN, *trans*-Golgi network.

Synaptotagmin (Syt) is a putative membrane trafficking protein defined as having an N-terminal single transmembrane domain and C-terminal tandem C2 Ca^{2+}-binding motifs (reviewed in Refs. *1–5*). To date, 15 distinct *syt* genes (*syt I–XV*) have been identified in mammals (*5–8*), and several *syt* genes have been identified in invertebrates and plants (*6*, *7*, *9*). Several Syt isoforms have been shown to control certain types of Ca^{2+}-regulated exocytosis. As an example, Syt I, the best characterized Syt isoform and abundant on synaptic vesicles, is now widely believed to function as the major Ca^{2+}-sensor for neurotransmitter release (*10–12*), and in the regulation of synaptic vesicle docking, fusion, and recycling steps via its C2 domains (*13–17*). Syts I, V, and IX are present on dense-core vesicles in some endocrine cells and regulate their Ca^{2+}-dependent exocytosis (*18–26*). Syt VI is involved in Ca^{2+}-dependent acrosome reactions in sperm cells (*27*), and Syt VII regulates Ca^{2+}-dependent lysosomal exocytosis in fibroblasts (*28–30*). However, the precise subcellular localizations and functions of other Syt isoforms (especially Syts III, IV, and VII in endocrine cells) are still matters of controversy (*22*, *31–36*).

Among the Syt family members, Syt IV (*37*) and Syt X have been suggested to be involved in synaptic plasticity, because their mRNA is rapidly increased by membrane depolarization in the brain (so-called "immediate early genes") (*38–41*). Consistent with this, Syt IV null mutant mice exhibit abnormalities in motor performance and some forms of memory related to the hippocampus (*42*). However, the precise role of the endogenous Syt IV protein in secretory vesicle exocytosis, that is, whether it is a positive or a negative regulator for exocytosis, remains to be elucidated (*43–47*), and even its localization is still a matter of controversy (synaptic vesicles, immature secretory vesicles, or dense-core vesicles) (*32*, *36*, *40*, *44*, *47–51*). Such discrepancies are most likely attributable to the use of recombinant protein transiently overexpressed without assessing the localization of endogenous Syt IV proteins, and the transient overexpression of recombinant Syt proteins has actually been found often to cause miss-localization or aggregation of Syt proteins in living cells (*22*, *35*, *52*). Using a specific anti-Syt IV antibody, we have recently shown that the Syt IV protein in undifferentiated PC12 cells is mainly localized in the Golgi and immature secretory vesicles, and that nerve

*To whom correspondence should be addressed. Tel: +81-48-462-4994, Fax: +81-48-462-4995, E-mail: mnfukuda@brain.riken.go.jp

 © 2004 The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

growth factor (NGF) treatment induces sorting of Syt IV protein to mature dense-core vesicles that undergo Ca^{2+}-dependent exocytosis (*36*). It would therefore be interesting to determine whether the membrane-depolarizing stimulus (or forskolin treatment) also promotes Syt IV protein sorting to mature, fusion-competent secretory vesicles; however, the sorting mechanism of newly synthesized Syt IV protein has never been determined.

In this study we demonstrated that forskolin treatment dramatically increases the endogenous Syt IV protein level (approximately 10-fold) in PC12 cells to a level comparable to that of the endogenous Syt IX protein, and that it promotes Syt IV protein sorting from the Golgi to the cell periphery (*i.e.*, directly beneath the plasma membrane) or to dense-core vesicles. Based on our findings, we discuss the possible role of the Syt IV protein in stimulus-dependent membrane trafficking.

MATERIALS AND METHODS

Materials—Anti-Rab3A, anti-Rab27A, anti-Syt IX (previously called Syt V) (*6*), anti-Munc18-1, and anti-GM130 mouse monoclonal antibodies were obtained from BD Transduction Laboratories (Lexington, KY, USA). Anti-Syt I (SYA148), anti-VAMP-2/synaptobrevin-2, and anti-BiP mouse monoclonal antibodies were purchased from StressGen Biotechnologies Corp. (Victoria, British Columbia). Anti-synaptophysin, brefeldin A (BFA), cytochalasin D, forskolin, and nocodazole were from Sigma Chemical Co. (St. Louis, MO, USA). Anti-syntaxin I/HPC-1 mouse monoclonal and anti-actin goat polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-transferrin receptor and anti-SNAP-25 mouse monoclonal antibodies were from Chemicon International Inc. (Temecula, CA, USA) and Upstate Biotechnology, Inc. (Lake Placid, NY, USA), respectively. Texas Red–conjugated phalloidin, Alexa Fluor 568–labeled anti-mouse IgG, and Alexa Fluor 488–labeled anti-rabbit IgG were obtained from Molecular Probes Inc. (Eugene, OR, USA). Wortmannin was from Calbiochem-Novachem Corp. (La Jolla, CA, USA). Anti-Syt IV-C2A rabbit polyclonal antibody was prepared as described previously (*40*). The purified anti-Syt IV-C2A antibody was conjugated with carboxyfluorescein (Molecular Probes Inc.) according to the manufacturer's instructions (*21*, *36*).

Cell Culture, Drug Treatment, and Immunocytochemistry—PC12 cells were cultured on glass-bottom-dishes (35-mm dish; MatTek Corp.; Ashland, MA, USA) coated with collagen type IV as described previously (*40*, *53*). PC12 cells were treated for 5 h with 50 μM forskolin with or without one of the following drugs: 50 nM wortmannin, 10 μg/ml BFA, 5 μM cytochalasin D, or 3.3 μg/ml nocodazole (*40*). The cells were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 20 min at room temperature, permeabilized with 0.3% Triton X-100 in PBS (phosphate-buffered saline) for 2 min, and incubated with the blocking solution (1% bovine serum albumin and 0.1% Triton X-100 in PBS) for 1 h at room temperature as described previously (*40*, *53*). The cells were then incubated with primary antibodies, anti-Syt IV-C2A rabbit polyclonal antibody (5 μg/ml) and anti-Rab27A (1/100 dilution), anti-Syt I (1/250 dilution), anti-transferrin receptor (1/100 dilution), anti-GM130 (1/100 dilution), anti-BiP mouse monoclonal antibody (1/200 dilution), or Texas Red-conjugated phalloidin (1/200 dilution). After washing the cells with blocking solution, the cells were incubated with secondary antibodies: Alexa Fluor 488–labeled anti-rabbit IgG (1/5,000 dilution) and Alexa Fluor 568–labeled anti-mouse IgG (1/5,000 dilution) for 1 h at room temperature. The cells were then analyzed under a confocal fluorescence microscope (Fluoview; Olympus, Tokyo, Japan). Images were further processed with Adobe Photoshop software (version 7.0).

Antibody-Uptake Experiments—After treatment with forskolin for 5 h (or with NGF for 2 d), PC12 cells were incubated for 15 min at 37°C with the rhodamine-labeled anti-Syt IV-N rabbit polyclonal antibody (10 μg/ml) in either high-KCl buffer (56 mM KCl, 95 mM NaCl, 2.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, and 15 mM HEPES-KOH, pH 7.4) or low-KCl buffer (5.6 mM KCl, 145 mM NaCl, 2.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, and 15 mM HEPES-KOH, pH 7.4) as described previously (*21*, *22*, *36*). The cells were immediately washed twice with PBS and then fixed with 4% paraformaldehyde as described above. Incorporated anti-Syt IV-N rabbit antibody was analyzed by confocal fluorescence microscopy as described above. Images were captured at random ($n > 45$), and dotted Syt IV signals were counted (see Fig. 5, B–D). Non-specific antibody-uptake was determined with the fluorescein-labeled anti-Syt IV-C2A antibody (10 μg/ml), which recognized the cytoplasmic domain of Syt IV, as a negative control.

Immunoblotting—Total homogenates of PC12 cells that had been treated or not treated with forskolin were prepared as described previously (*40*). A 30-μg sample of total cell lysate was subjected to 12.5% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore Corp.; Bedford, MA, USA). The blots were then probed with anti-Syt I (1/250 dilution), anti-Syt IV-C2A (6 μg/ml), anti-Syt IX (1/250 dilution), anti-Rab3A (1/100 dilution), anti-Rab27A (1/250 dilution), anti-syntaxin I (1/100 dilution), anti-SNAP-25 (1/1,000 dilution), anti-VAMP-2 (1/1,000 dilution), anti-Munc18-1 (1/1,000 dilution), anti-synaptophysin (1/1,000 dilution), and anti-actin antibodies (1/500 dilution) as described previously (*54*, *55*). Immunoreactive bands were visualized with enhanced chemiluminescence (Amersham Biosciences; Buckinghamshire, UK). The intensity of the bands on x-ray film was quantified with Lane Analyzer software (version 3.0) (ATTO Corp., Tokyo, Japan) and calibrated with T7-tagged recombinant proteins (T7-Syt IX or T7-Syt IV) as described previously (*21*, *56*, *57*). The blots shown in this paper are representative of two independent experiments.

Immunoelectron Microscopy—Immunoelectron microscopic analysis of endogenous Syt IV protein in PC12 cells was performed using anti-Syt IV-C2A rabbit polyclonal antibody essentially as described previously (*36*).

RESULTS

Forskolin Specifically Induces Syt IV Protein, but Not Other Proteins Involved in Regulated Exocytosis in PC12 Cells—In a previous study, we found that the Syt IV protein expression level in PC12 cells dramatically increased after treatment with forskolin, which can induce the late

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

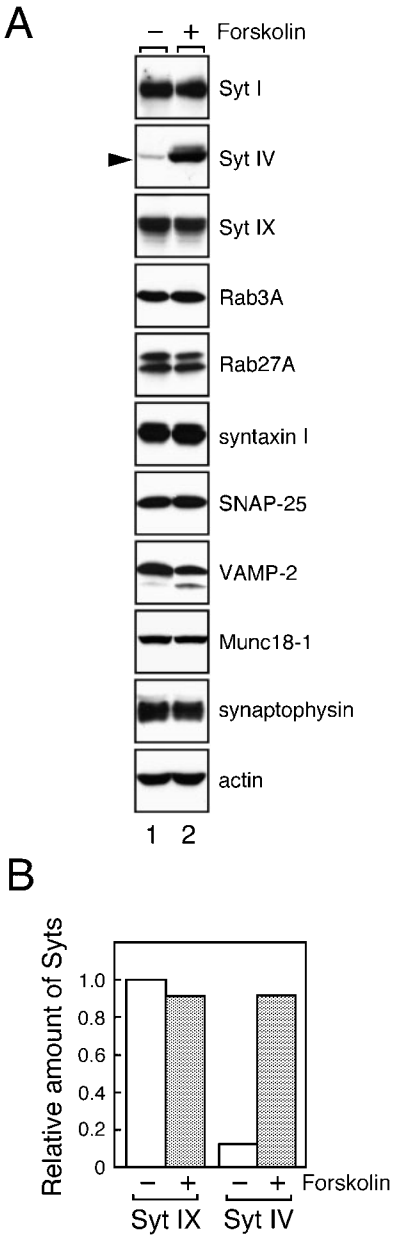


Fig. 1. **Specific induction of the Syt IV protein by forskolin treatment in PC12 cells.** (A) Forskolin-dependent increase in Syt IV protein in PC12 cells. PC12 cells were stimulated with 50 μM forskolin as described previously (*40*). Total homogenates of cells (30 μg) were subjected to 12.5% SDS-PAGE, transferred to a PVDF membrane, and then immunoblotted with the antibodies indicated. Note that the Syt IV protein level was dramatically increased by forskolin treatment (compare lanes 1 and 2 in the second panel), whereas the expression levels of other proteins involved in regulated exocytosis (*e.g.*, Syts I and IX) were unaltered by forskolin treatment. (B) Relative amount of Syt IX and Syt IV with (open bars) and without 50 μM forskolin treatment (shaded bars). The intensity of the bands on X-ray film was quantified with Lane Analyzer software (version 3.0) (ATTO Corp., Tokyo, Japan) and calibrated with the same amount of T7-tagged recombinant Syt proteins (T7-Syt IX and T7-Syt IV) as described previously (*21*). Forskolin induced approximately a 10-fold increase in the Syt IV protein level. The results shown are representative of two independent experiments.

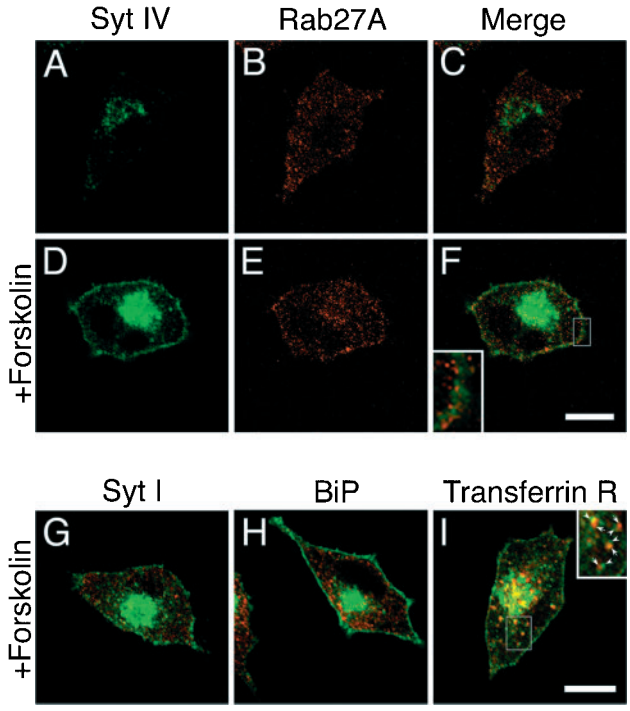


Fig. 2. **Distinct subcellular localization of the synaptotagmin IV protein and conventional secretory vesicle markers (Syt I and Rab27A) in PC12 cells.** After treatment with 50 μM forskolin (D–I) or without forskolin (A–C), PC12 cells were fixed, permeabilized, and stained with anti-Syt IV rabbit polyclonal antibody (5 μg/ml; green in A, D, G, H, and I), and anti-Rab27A (1/100 dilution; red in B and E), anti-Syt I (1/250 dilution; red in G), anti-BiP (1/200 dilution; red in H), or anti-transferrin receptor mouse monoclonal antibody (1/100 dilution; red in I). C and F are superpositions of A and B, and D and E, respectively. Note that an increase in Syt IV protein expression was evident in the Golgi as well as at the cell periphery (or near the plasma membrane) but that the Syt IV protein did not colocalize with the conventional secretory vesicle markers (see inset in F) even at the cell periphery. Scale bar = 20 μm.

phase of long-term potentiation (LTP) in the hippocampus (*40*). The forskolin-dependent up-regulation of the protein level seemed to be specific for Syt IV, because the protein expression levels of other proteins involved in regulated exocytosis (Syt I, Syt IX, Rab3A, Rab27A, syntaxin I, SNAP-25, VAMP-2, Munc18-1, and synaptophysin) were not altered very much by forskolin treatment (arrowhead in the second panel of Fig. 1A). Quantitative analysis indicated an approximate 10-fold increase in the Syt IV protein level after forskolin treatment (compare open and shaded bars in Fig. 1B), resulting in a Syt IV protein level almost identical to that of Syt IX, a major Syt isoform that regulates dense-core vesicle exocytosis in PC12 cells (*21*, *26*, *57*). This result, together with the fact that cAMP enhances hormone secretion from PC12 cells (*58*), prompted us to investigate whether the newly synthesized Syt IV protein is sorted to mature, fusion-competent secretory vesicles in forskolin-treated PC12 cells, which would contribute to an enhancement of regulated secretion.

Forskolin Induces Syt IV Protein-Sorting from the Golgi to the Cell Periphery in PC12 Cells—In the next set of experiments, we attempted to determine the subcellu-

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

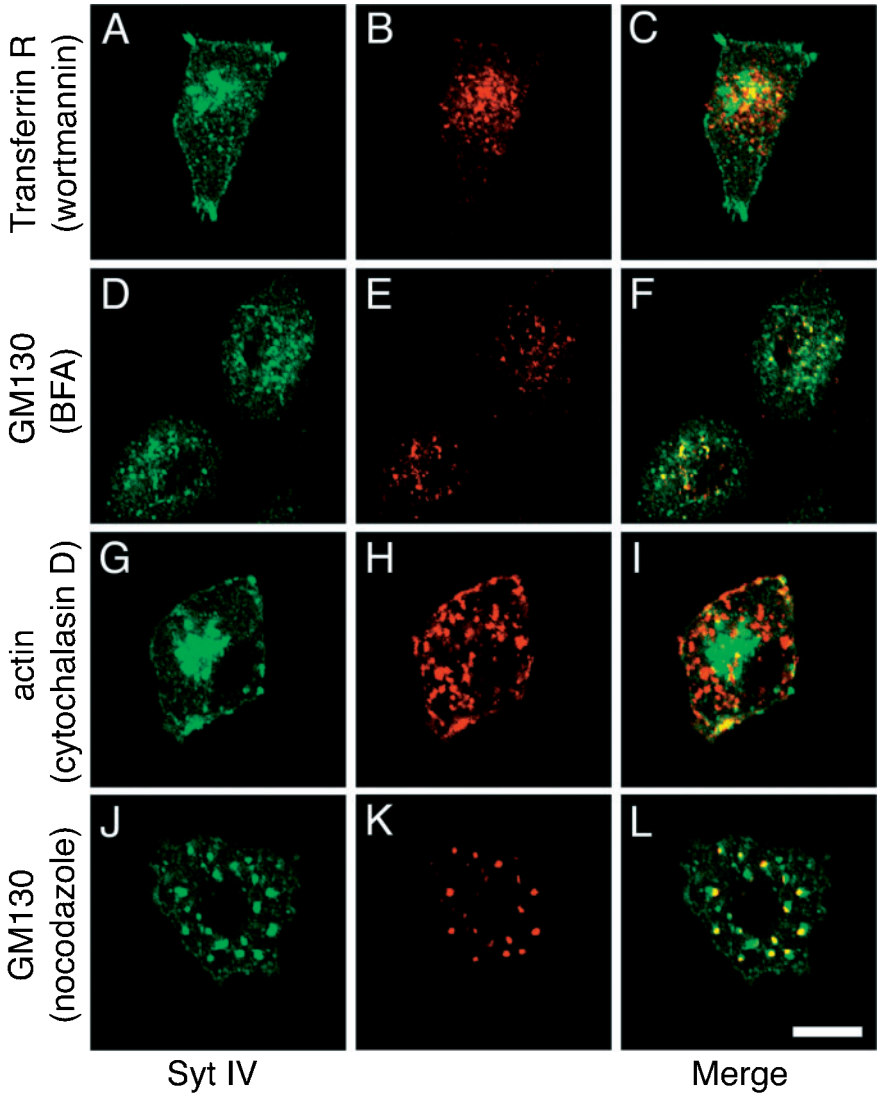


Fig. 3. **Forskolin enhances transport of the synaptotagmin IV protein from the Golgi to the cell periphery.** PC12 cells were treated with 50 μM forskolin together with the drugs indicated (50 nM wortmannin, 10 μg/ml BFA, 5 μM cytochalasin D, or 3.3 μg/ml nocodazole). PC12 cells were fixed, permeabilized, and stained with anti-Syt IV-C2A rabbit polyclonal antibody (green in A, D, G, and J), anti-transferrin receptor (1/100 dilution; red in B), anti-GM130 mouse monoclonal antibody (1/100 dilution; red in E and K), or Texas Red-conjugated phalloidin (1/200 dilution; red in H). C, F, I, and L are superpositions of A and B, D and E, G and H, and J and K, respectively. Note that BFA (disruption of the Golgi structure; see E) or nocodazole treatment (disruption of the microtubule network) dramatically reduced Syt IV immunostaining at the cell periphery (D and J), whereas wortmannin (alteration of endosome morphology; see B) or cytochalasin D treatment (disruption of actin filaments; see H) had virtually no effect on Syt IV localization. The Syt IV protein still colocalized with GM130, a Golgi marker, even after treatment with BFA (yellow in F) or nocodazole (yellow in L), consistent with the fact that Syt IV protein is mainly localized in the Golgi (*36*, *40*, *50*, *51*). Scale bar = 20 μm.

lar localization of the Syt IV protein in PC12 cells after forskolin treatment, especially focusing on dense-core vesicles. Under non-stimulated conditions, the Syt IV protein was mainly localized in the Golgi and immature secretory vesicles at the cell periphery (green in Fig. 2A) and did not colocalize with Rab27A, a dense-core vesicle marker (Fig. 2C) (*59*), consistent with our previous report (*36*, *40*). After forskolin treatment, however, Syt IV signals were dramatically increased in the Golgi, and Syt IV signals had accumulated at the periphery of most cells (77.3 ± 3.2% of the cells; n = 225) (green in Fig. 2D). Surprisingly, virtually no Syt IV signals (green in Fig. 2F and G) colocalized with the known secretory vesicle markers (Rab27A and Syt I; red in Fig. 2, F and G, respectively), even at the cell periphery of forskolin-treated PC12 cells (inset in Fig. 2F). It should be noted that the dense-core vesicle markers (red dots) were often localized outside the green Syt IV signals at the cell periphery (inset in Fig. 2F), suggesting that the Syt IV protein is likely to be present beneath the plasma membrane and not on the plasma membrane. This was also evident when cells were stained with anti-Syt IV N-terminal antibody without permeabilization with Triton X-100. The anti-Syt IV-N antibody failed to stain the plasma membrane of forskolin-treated PC12 cells (*36*) (see also Fig. 5B).

Since Syt IV signals in the cytoplasm increased after forskolin treatment, we compared the dotted Syt IV signals (green in Fig. 2H) in the cytoplasm with an ER (endoplasmic reticulum) marker, BiP (red in Fig. 2H), in forskolin-treated PC12 cells to determine whether the newly synthesized Syt IV protein is retained at the ER. As shown in Fig. 2H, Syt IV did not overlap with BiP, suggesting that newly synthesized Syt IV protein induced by forskolin is rapidly transported from the ER to the Golgi rather than being retained at the ER. Since the Syt IV signals in the forskolin-treated PC12 cells also did not overlap with an endosome marker, the transferrin receptor (red in inset of Fig. 2I), the dotted Syt IV signals in the cytoplasm may correspond to the transport vesicles from the Golgi to the cell periphery rather than to endosomes.

To investigate further the origin of Syt IV signals at the cell periphery, we treated cells with brefeldin A (BFA), which caused the destruction of the Golgi mor-

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

phology (GM130 in Fig. 3E) and re-distribution of some Golgi proteins to the ER, and thereby inhibited post-Golgi trafficking events, together with forskolin. It should be noted that the accumulation of Syt IV signals at the cell periphery was completely abolished by BFA treatment (Fig. 3D), but that some Syt IV signals still colocalized well with GM130 even after BFA treatment (yellow in Fig. 3F). By contrast, wortmannin, which altered the morphology of the endosomes but not of the Golgi or TGN (*trans*-Golgi network), had almost no effect on Syt IV protein localization (Fig. 3, A–C), consistent with the fact that the Syt IV protein is not localized in endosomes (Fig. 2I) (*36*, *40*). Interestingly, nocodazole, which disrupts microtubules, dramatically reduced the Syt IV signals at the cell periphery (Fig. 3J), whereas cytochalasin D, which disrupts actin filaments, had almost no effect on Syt IV localization (Fig. 3, G–I). Although nocodazole treatment also disrupted the Golgi structure (Fig. 3K), the majority of the Syt IV signals still colocalized with GM130 (yellow in Fig. 3L), consistent with the fact that Syt IV is a Golgi-resident protein (*36*, *51*). These results strongly indicate that forskolin induced Syt IV protein transport from the Golgi to the cell periphery (immediately adjacent to the plasma membrane) through microtubule-dependent movement rather than actin-based movement.

Forskolin Partially Promotes Sorting of Syt IV Protein to Secretory Vesicles That Undergo Ca^{2+}-Dependent Exocytosis in PC12 Cells—Although hardly any Syt IV protein colocalized with the known secretory vesicle markers at the immunocytochemical level (Fig. 2, F and G), it is still possible that a small amount of Syt IV protein is present on newly formed dense-core vesicles, which often lack Syt I or Syt IX proteins, in response to extracellular stimuli (*e.g.*, NGF and forskolin) (*36*). To test this possibility, immunoelectron microscopic analysis was performed using anti-Syt IV specific antibody (*36*, *40*). As anticipated, some, but not all, of the immuno-gold signals corresponding to the Syt IV molecule were clearly associated with dense-core vesicles (arrowheads and small arrows in Fig. 4A indicate Syt IV–positive and Syt IV–negative dense-core vesicles, respectively). Syt IV signals were often found on certain clear vesicular structures at the cell periphery (large arrows in Fig. 4A). Immuno-gold signals were also abundantly localized on the membranes of the Golgi in the cell body (Fig. 4B), consistent with the immunocytochemical results described above (Fig. 2D).

In the final set of experiments we attempted to determine whether the Syt IV–containing vesicles that form after forskolin treatment are capable of exocytosis in response to Ca^{2+}-stimulation. To this end, we performed an N-terminal antibody-uptake experiment as described previously (*21*, *22*, *36*) and visualized the dynamics of endogenous Syt IV molecules during Ca^{2+}-dependent exocytosis (see a schematic diagram in Fig. 5A for details). In brief, PC12 cells were stimulated with a high-KCl buffer for 15 min in the presence of antibodies against the luminal domain of Syt IV (anti-Syt IV-N), or antibodies against the cytoplasmic domain of Syt IV (anti-Syt IV-C2A, a negative control) in the extracellular medium. Incorporated antibodies were then analyzed by fluorescence microscopy, and dotted Syt IV signals were counted

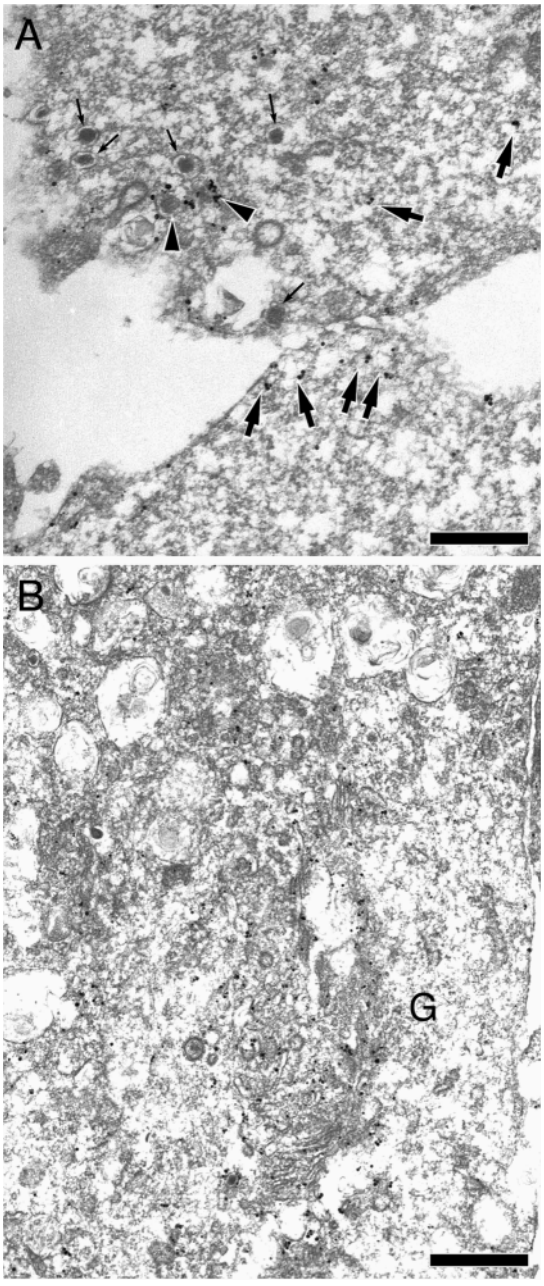
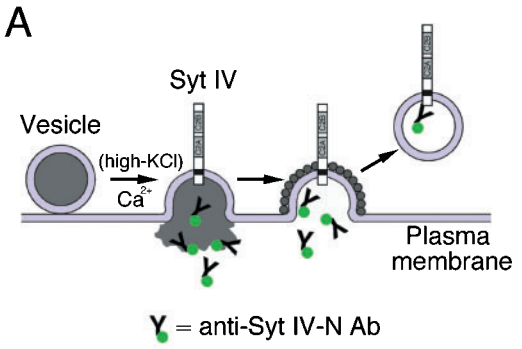


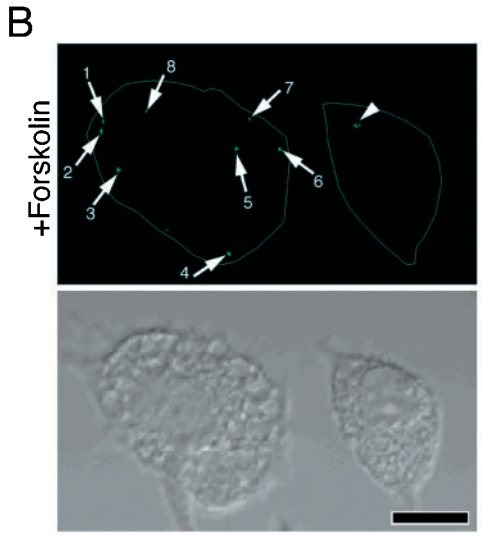
Fig. 4. **Localization of the Syt IV protein by immunoelectron microscopy in PC12 cells treated with forskolin.** Panels show representative views of the dense-core vesicle localization (A) and the Golgi localization (B) of Syt IV molecule stained with anti-Syt IV specific antibody followed by silver enhancement. Note that some, but not all, Syt IV signals (*i.e.*, gold particles) were clearly associated with dense-core vesicles at the cell periphery (arrowheads in A) or certain clear vesicular structures (large arrows in A). The small arrows indicate Syt IV-negative dense-core vesicles. Scale bars = 500 nm. G, Golgi.

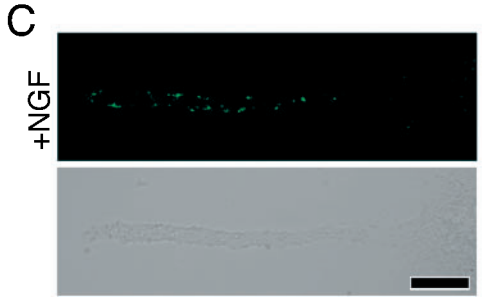
at random (Fig. 5, B–D). Significantly higher uptake of anti-Syt IV-N antibody was observed after forskolin treatment (2.80 ± 2.67 spots/cell; range: 0 to 11 spots; more than 80% of the cells took up antibodies) than under control conditions (0.98 ± 1.06 spots/cell; range: 0 to 4 spots; approximately 40% of the cells did not take up antibodies; and these values are almost the same as the

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

values for the non-specific uptake of the anti-Syt IV-C2A antibody) (see Fig. 5D). However, since the uptake of the anti-Syt IV-N antibody in forskolin-treated PC12 cells was much lower than in NGF-differentiated PC12 cells (22.5 ± 13.8 spots/neurite) (Fig. 5, C and D), we conclude that forskolin (or the increased cAMP level) alone is insufficient to sort all Syt IV molecules to mature, fusion-competent dense-core vesicles, and that additional factor(s) (*e.g.*, NGF) must be required for this process (*36*).







D

	Control	Forskolin-treated	NGF-treated
Number of Syt IV-N signals	0.98 ± 1.06 (n = 62)	2.80 ± 2.67* (n = 49)	22.50 ± 13.85* (n = 18)
Number of Syt IV-C2A signals (mean ± SD)	0.52 ± 0.77 (n = 60)	0.68 ± 1.16 (n = 84)	ND

*$p < 0.001$. ND, not determined.

DISCUSSION

In our previous studies we demonstrated that endogenous Syt IV molecules in undifferentiated PC12 cells are mainly localized in the Golgi, not on mature secretory vesicles (*36*, *40*), and that differentiation of PC12 cells with NGF is required for sorting the Syt IV protein to mature dense-core vesicles. In the present study we found that forskolin is also an important factor in stimulating transport of the Syt IV protein from the Golgi to the cell periphery or to dense-core vesicles in PC12 cells in a fashion different from NGF. As summarized in Fig. 6, we postulated four distinct stages of transport of Syt IV molecules (stages [I] to [IV]). In the initial stage, newly synthesized Syt IV molecules are rapidly recruited from the ER and retained at the Golgi, possibly via the Golgi localization signal in the spacer domain of Syt IV (*36*, *40*, *50*). Although the level of expression of the Syt IV protein in PC12 cells is usually very low (less than 5% that of Syt I) (*57*), forskolin rapidly stimulates the transcription of the Syt IV mRNA, possibly in a PKA- and CREB (cAMP-responsive element–binding protein)-dependent manner (*38*, *60*), and the resulting Syt IV protein level is approximately ten times greater than in the unstimulated condition (Fig. 1B) (*40*). Since Syt IV did not colocalize with the ER marker BiP, even after forskolin treatment (Fig. 2H), the newly formed Syt IV molecule must be rapidly targeted to the Golgi, resulting in the dramatic increase in Syt IV signals in the Golgi at the immunocytochemical (compare Fig. 2A and D) and immunoelectron microscopic levels (Fig. 4B). By contrast, NGF does not alter the total Syt IV protein level (*40*). In the second stage, a very small population of Syt IV molecules is constitutively transported from the Golgi to the cell periphery, especially into the cellular processes (Fig. 2A) (*36*), by a microtubule-dependent motor(s). This post-Golgi trafficking event was strongly inhibited by nocodazole (Fig. 3J) and BFA (Fig. 3D). Although forskolin greatly promotes the post-Golgi trafficking of Syt IV molecules (accumulation of Syt IV signals directly beneath the plasma membrane; Fig. 2D), most of the Syt IV molecules are present on clear vesicular structures (arrows in Fig.

Fig. 5. **Ca^{2+}-dependent uptake of anti-Syt IV-N antibody in forskolin-treated PC12 cells.** (A) Schematic representation of N-terminal antibody-uptake experiments. When a Syt IV–containing vesicle undergoes exocytosis in response to Ca^{2+}-stimulation (*i.e.*, high-KCl-stimulation), the luminal (or N-terminal) domain of Syt IV is transiently exposed to the extracellular medium and is recognized by the antibody against the luminal domain of Syt IV in the culture medium. The Syt IV·antibody complex is then incorporated into the cell by endocytosis. Incorporation of the antibody is strictly regulated by Ca^{2+}, not by membrane depolarization (*21*, *22*, *36*). (B and C) High-KCl–dependent uptake of the anti-Syt IV-N antibody in PC12 cells after forskolin (B) or NGF treatment (C) was measured as described previously (*21*, *22*, *36*). Incorporated anti-Syt IV-N rabbit antibody (peudo-colored in green) was analyzed by confocal fluorescence microscopy (upper panels). Images were captured at random, and dotted Syt IV signals (arrows and arrowhead) were counted (see Fig. 5D). The lower panels are bright-field images of the upper panels. Scale bars = 10 μm. (D) Enhanced uptake of the anti-Syt IV-N antibody in forskolin-treated and NGF-treated PC12 cells. Non-specific incorporation of the antibody into cells was determined with the anti-Syt IV-C2A antibody, which recognizes the cytoplasmic domain of Syt IV, as a negative control.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

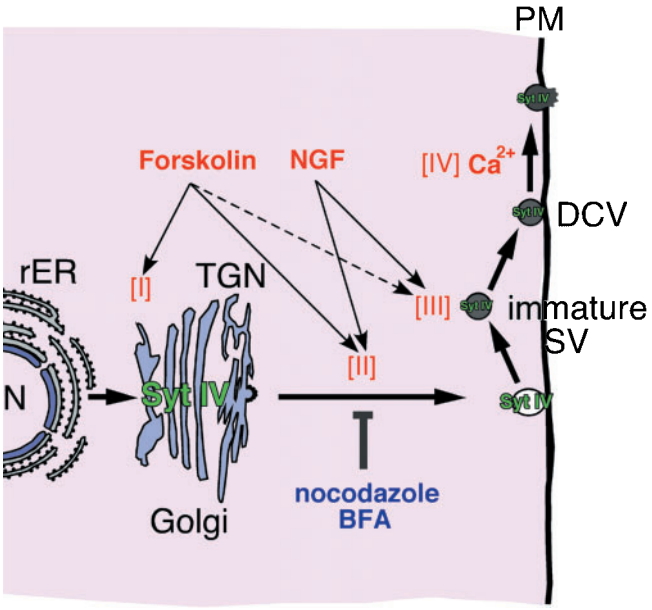


Fig. 6. **Model of synaptotagmin IV protein trafficking in PC12 cells.** The newly synthesized Syt IV protein is rapidly transported to and retained in the Golgi, possibly through the Golgi localization signal in the spacer domain of Syt IV (stage [I]) (*50*). A small population of Syt IV proteins is further transported from the Golgi to near the plasma membrane (PM) in undifferentiated PC12 cells (stage [II]) (*36*). Syt IV protein transport from the Golgi to the cell periphery requires microtubules rather than actin filaments, because nocodazole strongly inhibits this process while cytochalasin D does not (Fig. 3). Protein synthesis and transport of Syt IV protein to the cell periphery is significantly promoted by forskolin treatment (Figs. 1–3). Although forskolin partially induces the maturation of some secretory vesicles (*i.e.*, fusion-competent dense-core vesicles (DCV)) (Fig. 5), another factor(s) (*e.g.*, NGF) is required for the maturation of all DCVs (stage [III]) (*36*). Syt IV–containing DCVs undergo exocytosis in response to Ca^{2+}-stimulation (stage [IV]). N, nucleus, rER; rough ER; SV, secretory vesicles; and TGN, *trans*-Golgi network.

4A) and not further sorted to mature dense-core vesicles. These clear vesicles are unlikely to fuse to the plasma membrane in response to Ca^{2+}-stimulation, because uptake of the anti-Syt IV-N antibody was very limited in the forskolin-treated PC12 cells (Fig. 5D) in which many clear vesicular structures were found directly beneath the plasma membrane (Fig. 4A). Further work is needed to determine whether these clear vesicular structures correspond to immature secretory vesicles or secretory vesicles in a very primitive stage of formation as previously reported (*49*). NGF is also likely to promote the post-Golgi transport of Syt IV molecules, because many substances must be transported from the Golgi to the growing neurites during differentiation. In the third stage, Syt IV-containing vesicles mature into fusion-competent dense-core vesicles (or the Syt IV protein is sorted to mature dense-core vesicles). This maturation process is the rate-limiting step in Syt IV protein trafficking in PC12 cells, because we have often observed an accumulation of clear vesicular structures directly beneath the plasma membrane (large arrows in Fig. 4A), and only a small amount of Syt IV protein on dense-core vesicles (arrowheads in Fig. 4A) after forskolin treatment. By contrast, NGF stimulates maturation of dense-core vesicles much more than forskolin does (Fig. 5D) (*36*). In the final stage, Syt IV contributes to hormone secretion in response to Ca^{2+}-stimulation (stage [IV]), possibly by functioning as a Ca^{2+}-sensor (*61–63*).

Although Syt IV does not colocalize with Syt I in forskolin-treated PC12 cells at immunocytochemically detectable levels (Fig. 2G), a small fraction of Syt IV protein is clearly associated with some (but not all) dense-core vesicles in amounts that are detectable by immunoelectron microscopy (Fig. 4A), and similar observations have been reported in NGF-differentiated PC12 cells (*36*). This discrepancy may be explained by the notion that dense-core vesicles are heterogeneous with regard to the presence of Syt isoforms (Syts I/IX *versus* Syt IV) (*26*, *36*). Consistent with this notion, we recently found that the endogenous Syt IV protein is not co-purified with either Syt I– or Syt IX–containing vesicles (*26*). We, therefore, speculate that Syts I and IV may be sorted to distinct populations of secretory vesicles and control their exocytosis in forskolin-treated or NGF-differentiated PC12 cells, although the molecular mechanism of Syt I sorting to dense-core vesicles remains to be elucidated.

In summary, we have shown that forskolin treatment dramatically increases the Syt IV protein expression level (approximately 10-fold) and partially stimulates Syt IV protein sorting to dense-core vesicles in PC12 cells. At present, however, it is unclear whether this stimulus-dependent formation of Syt IV–containing secretory vesicles occurs in neurons under physiological conditions. Since tetanic stimulation activates both LTP and type I adenylyl cyclase in the brain (mimics forskolin-induced LTP) (*64*), strong synaptic activity may increase the intracellular cAMP level through activation of type I adenylyl cyclase, which increases the Syt IV protein level and promotes the formation of Syt IV–containing secretory vesicles in combination with NGF or neurotrophin(s). Further work is needed to determine whether newly formed Syt IV–containing secretory vesicles contribute to the plastic pre- and/or postsynaptic changes in the brain.

We would like to thank Eiko Kanno and Yukie Ogata for expert technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, and Technology of Japan (15689006 and 16044248 to M.F.).

REFERENCES

1. Schiavo, G., Osborne, S.L., and Sgouros, J.G. (1998) Synaptotagmins: more isoforms than functions? *Biochem. Biophys. Res. Commun.* **248**, 1–8
2. Marquèze, B., Berton, F., and Seagar, M. (2000) Synaptotagmins in membrane traffic: which vesicles do the tagmins tag? *Biochimie* **82**, 409–420
3. Chapman, E.R. (2002) Synaptotagmin: a Ca^{2+} sensor that triggers exocytosis? *Nat. Rev. Mol. Cell Biol.* **3**, 498–508
4. Südhof, T.C. (2002) Synaptotagmins: why so many? *J. Biol. Chem.* **277**, 7629–7632
5. Fukuda, M. (2003) Synaptotagmins, Ca^{2+}- and phospholipid-binding proteins that control Ca^{2+}-regulated membrane trafficking. *Recent Res. Dev. Chem. Phys. Lipids* **1**, 15–51
6. Craxton, M. (2001) Genomic analysis of synaptotagmin genes. *Genomics* **77**, 43–49

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

7. Fukuda, M. (2003) Molecular cloning, expression, and characterization of a novel class of synaptotagmin (Syt XIV) conserved from *Drosophila* to humans. *J. Biochem.* **133**, 641–649
8. Fukuda, M. (2003) Molecular cloning and characterization of human, rat, and mouse synaptotagmin XV. *Biochem. Biophys. Res. Commun.* **306**, 64–71
9. Adolfsen, B. and Littleton, J.T. (2001) Genetic and molecular analysis of the synaptotagmin family. *Cell. Mol. Life Sci.* **58**, 393–402
10. Fernandez-Chacon, R., Konigstorfer, A., Gerber, S.H., Garcia, J., Matos, M.F., Stevens, C.F., Brose, N., Rizo, J., Rosenmund, C., and Südhof, T.C. (2001) Synaptotagmin I functions as a calcium regulator of release probability. *Nature* **410**, 41–49
11. Mackler, J.M., Drummond, J.A., Loewen, C.A., Robinson, I.M., and Reist, N.E. (2002) The C_2B Ca^{2+}-binding motif of synaptotagmin is required for synaptic transmission *in vivo*. *Nature* **418**, 340–344
12. Yoshihara, M. and Littleton, J.T. (2002) Synaptotagmin I functions as a calcium sensor to synchronize neurotransmitter release. *Neuron* **36**, 897–908
13. Bommert, K., Charlton, M.P., DeBello, W.M., Chin, G.J., Betz, H., and Augustine, G.J. (1993) Inhibition of neurotransmitter release by C2-domain peptides implicates synaptotagmin in exocytosis. *Nature* **363**, 163–165
14. Mikoshiba, K., Fukuda, M., Moreira, J.E., Lewis, F.M.T., Sugimori, M., Niinobe, M., and Llinás, R. (1995) Role of the C2A domain of synaptotagmin in transmitter release as determined by specific antibody injection into the squid giant synapse preterminal. *Proc. Natl. Acad. Sci. USA* **92**, 10703–10707
15. Fukuda, M., Moreira, J.E., Lewis, F.M.T., Sugimori, M., Niinobe, M., Mikoshiba, K., and Llinás, R. (1995) Role of the C2B domain of synaptotagmin in vesicular release and recycling as determined by specific antibody injection into the squid giant synapse preterminal. *Proc. Natl. Acad. Sci. USA* **92**, 10708–10712
16. Fukuda, M., Moreira, J.E., Liu, V., Sugimori, M., Mikoshiba, K., and Llinás, R.R. (2000) Role of the conserved WHXL motif in the C terminus of synaptotagmin in synaptic vesicle docking. *Proc. Natl. Acad. Sci. USA* **97**, 14715–14719
17. Chieregatti, E., Witkin, J.W., and Baldini, G. (2002) SNAP-25 and synaptotagmin 1 function in Ca^{2+}-dependent reversible docking of granules to the plasma membrane. *Traffic* **3**, 496–511
18. Lang, J., Fukuda, M., Zhang, H., Mikoshiba, K., and Wollheim, C.B. (1997) The first C2 domain of synaptotagmin is required for exocytosis of insulin from pancreatic β-cells: action of synaptotagmin at low micromolar calcium. *EMBO J.* **16**, 5837–5846
19. Ohara-Imaizumi, M., Fukuda, M., Niinobe, M., Misonou, H., Ikeda, K., Murakami, T., Kawasaki, M., Mikoshiba, K., and Kumakura, K. (1997) Distinct roles of C2A and C2B domains of synaptotagmin in the regulation of exocytosis in adrenal chromaffin cells. *Proc. Natl. Acad. Sci. USA* **94**, 287–291
20. Voets, T., Moser, T., Lund, P.E., Chow, R.H., Geppert, M., Südhof, T.C., and Neher, E. (2001) Intracellular calcium dependence of large dense-core vesicle exocytosis in the absence of synaptotagmin I. *Proc. Natl. Acad. Sci. USA* **98**, 11680–11685
21. Fukuda, M., Kowalchyk, J.A., Zhang, X., Martin, T.F.J., and Mikoshiba, K. (2002) Synaptotagmin IX regulates Ca^{2+}-dependent secretion in PC12 cells. *J. Biol. Chem.* **277**, 4601–4604
22. Saegusa, C., Fukuda, M., and Mikoshiba, K. (2002) Synaptotagmin V is targeted to dense-core vesicles that undergo calcium-dependent exocytosis in PC12 cells. *J. Biol. Chem.* **277**, 24499–24505
23. Tucker, W.C., Edwardson, J.M., Bai, J., Kim, H.J., Martin, T.F.J., and Chapman, E.R. (2003) Identification of synaptotagmin effectors via acute inhibition of secretion from cracked PC12 cells. *J. Cell Biol.* **162**, 199–209
24. Kreft, M., Kuster, V., Grilc, S., Rupnik, M., Milisav, I., and Zorec, R. (2003) Synaptotagmin I increases the probability of vesicle fusion at low $[Ca^{2+}]$ in pituitary cells. *Amer. J. Physiol. Cell Physiol.* **284**, C547–C554
25. Iezzi, M., Kouri, G., Fukuda, M., and Wollheim, C.B. (2004) Synaptotagmin V and IX isoforms control Ca^{2+}-dependent insulin exocytosis. *J. Cell Sci.* **117**, 3119–3127
26. Fukuda, M. (2004) RNA interference-mediated silencing of synaptotagmin IX, but not synaptotagmin I, inhibits dense-core vesicle exocytosis in PC12 cells. *Biochem. J.* **380**, 875–879
27. Michaut, M., De Blas, G., Tomes, C.N., Yunes, R., Fukuda, M., and Mayorga, L.S. (2001) Synaptotagmin VI participates in the acrosome reaction of human spermatozoa. *Dev. Biol.* **235**, 521–529
28. Martinez, I., Chakrabarti, S., Hellevik, T., Morehead, J., Fowler, K., and Andrews, N.W. (2000) Synaptotagmin VII regulates Ca^{2+}-dependent exocytosis of lysosomes in fibroblasts. *J. Cell Biol.* **148**, 1141–1149
29. Reddy, A., Caler, E.V., and Andrews, N.W. (2001) Plasma membrane repair is mediated by Ca^{2+}-regulated exocytosis of lysosomes. *Cell* **106**, 157–169
30. Chakrabarti, S., Kobayashi, K.S., Flavell, R.A., Marks, C.B., Miyake, K., Liston, D.R., Fowler, K.T., Gorelick, F.S., and Andrews, N.W. (2003) Impaired membrane resealing and autoimmune myositis in synaptotagmin VII-deficient mice. *J. Cell Biol.* **162**, 543–549
31. Mizuta, M., Kurose, T., Miki, T., Shoji-Kasai, Y., Takahashi, M., Seino, S., and Matsukura, S. (1997) Localization and functional role of synaptotagmin III in insulin secretory vesicles in pancreatic β-cells. *Diabetes* **46**, 2002–2006
32. Ferguson, G.D., Thomas, D.M., Elferink, L.A., and Herschman, H.R. (1999) Synthesis degradation, and subcellular localization of synaptotagmin IV, a neuronal immediate early gene product. *J. Neurochem.* **72**, 1821–1831
33. Gut, A., Kiraly, C.E., Fukuda, M., Mikoshiba, K., Wollheim, C.B., and Lang, J. (2001) Expression and localisation of synaptotagmin isoforms in endocrine β-cells: their function in insulin exocytosis. *J. Cell Sci.* **114**, 1709–1716
34. Sugita, S., Han, W., Butz, S., Liu, X., Fernandez-Chacon, R., Lao, Y., and Südhof, T.C. (2001) Synaptotagmin VII as a plasma membrane Ca^{2+} sensor in exocytosis. *Neuron* **30**, 459–473
35. Fukuda, M., Ogata, Y., Saegusa, C., Kanno, E., and Mikoshiba, K. (2002) Alternative splicing isoforms of synaptotagmin VII in the mouse, rat and human. *Biochem. J.* **365**, 173–180
36. Fukuda, M., Kanno, E., Ogata, Y., Saegusa, C., Kim, T., Peng Loh, Y., and Yamamoto, A. (2003) Nerve growth factor–dependent sorting of synaptotagmin IV protein to mature dense-core vesicles that undergo calcium-dependent exocytosis in PC12 cells. *J. Biol. Chem.* **278**, 3220–3226
37. Hilbush, B.S. and Morgan, J.I. (1994) A third synaptotagmin gene, *Syt3*, in the mouse. *Proc. Natl. Acad. Sci. USA* **91**, 8195–8199.
38. Vician, L., Lim, I.K., Ferguson, G., Tocco, G., Baudry, M., and Herschman, H.R. (1995) Synaptotagmin IV is an immediate early gene induced by depolarization in PC12 cells and in brain. *Proc. Natl. Acad. Sci. USA* **92**, 2164–2168
39. Babity, J.M., Armstrong, J.N., Plumier, J.C., Currie, R.W., and Robertson, H.A. (1997) A novel seizure-induced synaptotagmin gene identified by differential display. *Proc. Natl. Acad. Sci. USA* **94**, 2638–2641
40. Ibata, K., Fukuda, M., Hamada, T., Kabayama, H., and Mikoshiba, K. (2000) Synaptotagmin IV is present at the Golgi and distal parts of neurites. *J. Neurochem.* **74**, 518–526
41. Ferguson, G.D., Vician, L., and Herschman, H.R. (2001) Synaptotagmin IV: biochemistry, genetics, behavior, and possible links to human psychiatric disease. *Mol. Neurobiol.* **23**, 173–185
42. Ferguson, G.D., Anagnostaras, S.G., Silva, A.J., and Herschman, H.R. (2000) Deficits in memory and motor performance in synaptotagmin IV mutant mice. *Proc. Natl. Acad. Sci. USA* **97**, 5598–5603
43. Littleton, J.T., Serano, T.L., Rubin, G.M., Ganetzky, B., and Chapman, E.R. (1999) Synaptic function modulated by

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

changes in the ratio of synaptotagmin I and IV. *Nature* **400**, 757–760
44. Thomas, D.M., Ferguson, G.D., Herschman, H.R., and Elferink, L.A. (1999) Functional and biochemical analysis of the C2 domains of synaptotagmin IV. *Mol. Biol. Cell* **10**, 2285–2295
45. Wang, C.T., Grishanin, R., Earles, C.A., Chang, P.Y., Martin, T.F.J., Chapman, E.R., and Jackson, M.B. (2001) Synaptotagmin modulation of fusion pore kinetics in regulated exocytosis of dense-core vesicles. *Science* **294**, 1111–1115
46. Tsuboi, T. and Rutter, G.A. (2003) Multiple forms of "kiss-and-run" exocytosis revealed by evanescent wave microscopy. *Curr. Biol.* **13**, 563–567
47. Wang, C.T., Lu, J.C., Bai, J., Chang, P.Y., Martin, T.F.J., Chapman, E.R., and Jackson, M.B. (2003) Different domains of synaptotagmin control the choice between kiss-and-run and full fusion. *Nature* **424**, 943–947
48. Berton, F., Cornet, V., Iborra, C., Garrido, J., Dargent, B., Fukuda, M., Seagar, M., and Marquèze, B. (2000) Synaptotagmin I and IV define distinct populations of neuronal transport vesicles. *Eur. J. Neurosci.* **12**, 1294–1302
49. Eaton, B.A., Haugwitz, M., Lau, D., and Moore, H.P. (2000) Biogenesis of regulated exocytotic carriers in neuroendocrine cells. *J. Neurosci.* **20**, 7334–7344
50. Fukuda, M., Ibata, K., and Mikoshiba, K. (2001) A unique spacer domain of synaptotagmin IV is essential for Golgi localization. *J. Neurochem.* **77**, 730–740
51. Ibata, K., Hashikawa, T., Tsuboi, T., Terakawa, S., Liang, F., Mizutani, A., Fukuda, M., and Mikoshiba, K. (2002) Non-polarized distribution of synaptotagmin IV in neurons: evidence that synaptotagmin IV is not a synaptic vesicle protein. *Neurosci. Res.* **43**, 401–406
52. Fukuda, M., Yamamoto, A., and Mikoshiba, K. (2001) Formation of crystalloid endoplasmic reticulum induced by expression of synaptotagmin lacking the conserved WHXL motif in the C terminus: structural importance of the WHXL motif in the C2B domain. *J. Biol. Chem.* **276**, 41112–41119
53. Fukuda, M. and Mikoshiba, K. (1999) A novel alternatively spliced variant of synaptotagmin VI lacking a transmembrane domain: implications for distinct functions of the two isoforms. *J. Biol. Chem.* **274**, 31428–31434
54. Fukuda, M., Kanno, E., and Mikoshiba, K. (1999) Conserved N-terminal cysteine motif is essential for homo- and heterodimer formation of synaptotagmins III, V, VI, and X. *J. Biol. Chem.* **274**, 31421–31427
55. Fukuda, M. (2003) Slp4-a/granuphilin-a inhibits dense-core vesicle exocytosis through interaction with the GDP-bound form of Rab27A in PC12 cells. *J. Biol. Chem.* **278**, 15390–15396
56. Fukuda, M. and Mikoshiba, K. (2000) Distinct self-oligomerization activities of synaptotagmin family: unique calcium-dependent oligomerization properties of synaptotagmin VII. *J. Biol. Chem.* **275**, 28180–28185
57. Zhang, X., Kim-Miller, M.J., Fukuda, M., Kowalchyk, J.A., and Martin, T.F.J. (2002) Ca^{2+}-dependent synaptotagmin binding to SNAP-25 is essential for Ca^{2+}-triggered exocytosis. *Neuron* **34**, 599–611
58. Rabe, C.S. and McGee, R., Jr. (1983) Regulation of depolarization-dependent release of neurotransmitters by adenosine: cyclic AMP–dependent enhancement of release from PC12 cells. *J. Neurochem.* **41**, 1623–1634
59. Fukuda, M., Kanno, E., and Yamamoto, A. (2004) Rabphilin and Noc2 are recruited to dense-core vesicles through specific interaction with Rab27A in PC12 cells. *J. Biol. Chem.* **279**, 13065–13075
60. Ferguson, G.D., Chen, X.N., Korenberg, J.R., and Herschman, H.R. (2000) The human synaptotagmin IV gene defines an evolutionary break point between syntenic mouse and human chromosome regions but retains ligand inducibility and tissue specificity. *J. Biol. Chem.* **275**, 36920–36926
61. Fukuda, M., Kojima, T., and Mikoshiba, K. (1996) Phospholipid composition dependence of Ca^{2+}-dependent phospholipid binding to the C2A domain of synaptotagmin IV. *J. Biol. Chem.* **271**, 8430–8434
62. Robinson, I.M., Ranjan, R., and Schwarz, T.L. (2002) Synaptotagmins I and IV promote transmitter release independently of Ca^{2+} binding in the C_2A domain. *Nature* **418**, 336–340
63. Zhang, Q., Fukuda, M., Van Bockstaele, E., Pascual, O., and Haydon, P.G. (2004) Synaptotagmin IV regulates glial glutamate release. *Proc. Natl. Acad. Sci. USA* **101**, 9441–9446
64. Salin, P.A., Malenka, R.C., and Nicoll, R.A. (1996) Cyclic AMP mediates a presynaptic form of LTP at cerebellar parallel fiber synapses. *Neuron* **16**, 797–803

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012