Ca^{2+}-dependent interaction of the growth-associated protein GAP-43 with the synaptic core complex

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The synaptic vesicle exocytosis occurs by a highly regulated mechanism: syntaxin and 25 kDa synaptosome-associated protein (SNAP-25) are assembled with vesicle-associated membrane protein (VAMP) to form a synaptic core complex and then synaptotagmin participates as a Ca^{2+} sensor in the final step of membrane fusion. The 43 kDa growth-associated protein GAP-43 is a nerve-specific protein that is predominantly localized in the axonal growth cones and presynaptic terminal membrane. In the present study we have examined a possible interaction of GAP-43 with components involved in the exocytosis. GAP-43 was found to interact with syntaxin, SNAP-25 and VAMP in rat brain tissues and nerve growth factor-dependently differentiated PC12 cells, but not in undifferentiated PC12 cells. GAP-43 also interacted with synaptotagmin and calmodulin. These interactions of GAP-43 could be detected only when chemical cross-linking of proteins was performed before they were solubilized from the membranes with detergents, in contrast with the interaction of the synaptic core complex, which was detected without cross-linking. Experiments *in vitro* showed that the interaction of GAP-43 with these proteins occurred Ca^{2+}-dependently; its maximum binding with the core complex was observed at 100 μM Ca^{2+}, whereas that of syntaxin with synaptotagmin was at 200 μM Ca^{2+}. These values of Ca^{2+} concentration are close to that required for the Ca^{2+}-dependent release of neurotransmitters. Furthermore we observed that the interaction *in vitro* of GAP-43 with the synaptic core complex was coupled with protein kinase C-mediated phosphorylation of GAP-43. Taken together, our results suggest a novel function of GAP-43 that is involved in the Ca^{2+}-dependent fusion of synaptic vesicles.

INTRODUCTION

GAP-43 (also called neuromodulin) is a 43 kDa nerve-specific protein associated with axonal growth cones for which several possible functions have been proposed [1,2]. Although it is highly expressed in neurons during development and regeneration [1,3], GAP-43 is still expressed in mature neurons predominantly on the internal surface of presynaptic terminal membranes [4,5]. It has been suggested that GAP-43 is involved in the neural signal transduction system, because it interacts with and activates the GTP-binding protein G_0 by a mechanism similar to that involving G-protein-coupled receptors [6,7]. GAP-43 is well known as a protein kinase C (PKC) substrate in neurons [8,9]. The PKC-mediated phosphorylation of GAP-43 is closely related to neurotransmitter release [10] and long-term potentiation [11]. Furthermore GAP-43 has a calmodulin-binding domain near the N-terminus and has been suggested to act as a calmodulin store at the nerve terminal membrane [12,13]. Because the calmodulin-binding domain also contains the PKC-phosphorylation site (Ser-41), the binding of calmodulin to GAP-43 is controlled by its phosphorylation in a reciprocal manner [14,15].

It was shown that the Ca^{2+}-induced neurotransmitter release is inhibited by anti-GAP-43 antibodies in permeabilized synaptosomes [16,17] and also by the expression of antisense GAP-43 RNA in PC12 cells [18]. Furthermore the introduction of GAP-43 peptide, which was characterized as interacting with granule-associated G_0 in chromaffin cells, was shown to control Ca^{2+}-regulated exocytosis [19]. These observations suggest that GAP-43 is involved in the Ca^{2+}-dependent neurotransmitter release, but the precise molecular mechanism for the modulation of exocytosis by GAP-43 remains to be clarified.

Synaptic vesicle exocytosis with neurotransmitter release occurs by a highly regulated mechanism at the active zone of presynaptic membrane and can be divided into several steps [20]. For docking of the vesicles to the membrane, the plasma membrane proteins syntaxin and 25 kDa synaptosome-associated protein (SNAP-25) are assembled with the vesicle-associated membrane protein VAMP (or synaptobrevin) to form a synaptic core complex that serves as a receptor for the *N*-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs) [21,22]. Thus these membrane proteins are designated SNAP receptors (SNAREs). The SNARE components are highly conserved in eukaryotic cells [23,24] and are essential for the synaptic vesicle exocytosis, because each of these components is selectively proteolysed by *Clostridium botulinum* neurotoxins and tetanus toxin, which are potent inhibitors of neurotransmitter release [25,26]. The final fusion step of synaptic vesicles is a Ca^{2+}-dependent event, in which synaptotagmin is implicated as participating as a low-affinity Ca^{2+} sensor [27], but the mechanisms and molecules that participate in this step have not been entirely characterized [28,29].

In contrast with constitutive exocytosis, synaptic vesicle exocytosis requires tight regulation: synaptic vesicles should be docked to the active zone of presynaptic membrane and fused by Ca^{2+} influx for accurate neurotransmission. In the present study we have investigated whether GAP-43 is a candidate for involvement in the vesicle docking/fusion machinery. We found that GAP-43 does indeed interact with the synaptic core complex

Abbreviations used: DSP, dithiobis(succinimidylpropionate); GAP-43, 43 kDa growth-associated protein; NGF, nerve growth factor; NSF, *N*-ethylmaleimide-sensitive fusion protein; PKC, protein kinase C; SNAP, soluble NSF attachment protein; SNAP-25, 25 kDa synaptosome-associated protein; SNARE, SNAP receptor; VAMP, vesicle-associated membrane protein.

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in a Ca^{2+}-dependent manner, suggesting that GAP-43 is involved in the Ca^{2+}-dependent fusion of synaptic vesicles. Moreover we analysed the effect of PKC-dependent phosphorylation of GAP-43 on the interaction.

MATERIALS AND METHODS

Materials

Nerve growth factor (NGF) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). RPMI 1640 medium was from Gibco-BRL (Grand Island, NY, U.S.A.); dithiobis(succinimidyl propionate) (DSP) from Pierce Chemical Co. (Rockford, IL, U.S.A.). Mouse monoclonal antibodies against SNAP-25, syntaxin and synaptotagmin, and rabbit polyclonal anti-VAMP2 antibodies, were obtained from Wako Chemicals (Osaka, Japan); sheep anti-calmodulin serum from Chemicon International (Temecula, CA, U.S.A.). Rhodamine-conjugated goat anti-(rabbit IgG), FluoroLink Cy2-labelled donkey anti-(mouse IgG), the enhanced chemiluminescence kit, [^{32}P]P_i and [γ-^{32}P]ATP were obtained from Amersham Corp. (Tokyo, Japan). PMA, and the PKC inhibitor H-7, were from Sigma (St. Louis, MO, U.S.A.).

Preparation of anti-GAP-43 antibody

The synthetic peptide (Cys-Lys-Glu-Ser-Ala-Arg-Gln-Asp-Glu-Gly-Lys-Glu-Asp-Pro-Gln-Ala-Asp-Gln-Glu-His-Ala) was used for raising antibodies in rabbits. The sequence corresponds to the C-terminal portion of rat GAP-43 (positions 207–226) [30]. This peptide (5 mg) was conjugated to keyhole limpet haemocyanin; the conjugates (300 μg/rabbit) were injected into two rabbits every 3 weeks as described previously [31,32]. Antisera obtained from the rabbits were subjected to affinity chromatography through a Sepharose column coupled with the peptide used as the antigen.

Cell culture and fractionation

PC12 cells were cultured in RPMI 1640 medium supplemented with penicillin (100 i.u./ml), streptomycin (100 μg/ml), fetal calf serum (5%, v/v) and horse serum (10%, v/v) in an air/CO_2 (19:1) atmosphere at 37 °C. When indicated, cells grown on poly-(L-lysine)-coated plastic dishes at a density of 1.5×10^6 cells per 35 mm dish were treated for 4 days with NGF (50 ng/ml) in RPMI 1640 medium containing 0.5% (v/v) fetal calf serum and 1% (v/v) horse serum. Control cells were incubated in the same manner in the absence of NGF. PC12 cells cultured for 4 days in the absence or presence of NGF were washed twice in Dulbecco's PBS and harvested with a scraper. After homogenization in 200 μl of buffer A [20 mM Hepes/NaOH (pH 7.4)/120 mM NaCl/4.5 mM KCl/1.3 mM $MgCl_2$] by 30 passages through a 27-gauge needle, cell debris was removed by centrifugation at 10000 ***g*** for 5 min. The homogenates were separated into cytosol and membrane fractions by centrifugation at 105000 ***g*** for 1 h.

Analysis of brain slices

A whole brain of an adult male Wistar rat (200–250 g) was dissected and sliced on ice. For analysis *in vivo*, the slices (100 mg wet weight of a whole brain per sample) were incubated at 37 °C for 30 min in 2 ml of buffer A in the presence of 5 mM EGTA or 2.5 mM Ca^{2+}. After being washed with ice-cold buffer A, the slices were immediately homogenized in 1 ml of the same buffer with a Teflon glass homogenizer (30 strokes) and centrifuged at 1000 ***g*** for 5 min. Equal amounts of the supernatant (100 μg of protein in 100 μl per tube) were immediately subjected to chemical cross-linking, when indicated, and then solubilized for immunoprecipitation, as described below. For analysis *in vitro*, homogenates were prepared from brain slices that had been incubated in the presence of 2.5 mM Ca^{2+} as above. Equal amounts of the homogenates (100 μg of protein in 100 μl per tube) were incubated at 37 °C for 30 min in buffer A containing the indicated concentrations of Ca^{2+} or 5 mM EGTA. Each sample was subjected to cross-linking followed by solubilization and immunoprecipitation.

Chemical cross-linking

Homogenates of PC12 cells and brain tissues were incubated at 4 °C for 30 min with DSP dissolved in DMSO (final DSP concentration 0.1 mM) for cross-linking of proteins. The remaining DSP was quenched by incubating the samples at 4 °C for 30 min with 50 mM Tris/HCl, pH 7.4. These samples were solubilized and used for immunoprecipitation. When indicated the homogenates were first solubilized with the indicated detergents and then treated with DSP.

Immunoprecipitation and immunoblotting

Homogenates of PC12 cells and brain tissues were adjusted to contain 1% (v/v) Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS in 0.5 ml of PBS. A cytosolic fraction was also adjusted to contain the same concentrations of the detergents. A protease-inhibitor mixture (antipain, chymostatin, elastinal, leupeptin, pepstatin A and phosphoramidon; final concentration of each was 10 μg/ml) was added to each sample. The samples were subjected to immunoprecipitation as described previously [31,32]. The lysates were centrifuged at 15000 ***g*** for 10 min and the resultant supernatants were incubated with the indicated antibodies at 4 °C overnight followed by the addition of 100 μl of 50% (v/v) Protein G–Sepharose 4FF (Pharmacia Biotech). After shaking at 4 °C for 1 h, the beads were washed extensively [31,32]. The immunoprecipitates were boiled for 4 min in 60 μl of SDS/PAGE sample buffer containing 5% (v/v) 2-mercaptoethanol and analysed by SDS/PAGE [10% (w/v) gel]. After separation by SDS/PAGE, the proteins were transferred to PVDF membrane (0.45 μm; Millipore). Membranes were blocked in 0.5% (w/v) Blocking reagent (Boehringer Mannheim) and incubated with the indicated antibodies for 1 h at room temperature. The horseradish peroxidase-conjugated sheep anti-(rabbit IgG) or anti-(mouse IgG) was used as a secondary antibody; incubation was for 1 h at room temperature. The immunoreactive bands were revealed with the enhanced chemiluminescence kit. The intensities of immunoreactive bands detected by enhanced chemiluminescence were quantified as pixel values by a Macintosh 8100 computer with the public-domain NIH image program.

Immunocytochemistry

PC12 cells grown on poly-L-lysine-coated glass coverslips were cultured for 4 days in RPMI 1640 medium in the absence or presence of 50 ng/ml NGF. Cells were washed in PBS and fixed in 4% (w/v) paraformaldehyde in PBS for 20 min at room temperature. After being washed twice in PBS, cells were permeabilized in 0.1% Triton X-100 in PBS for 20 min. For the double-immunofluorescence staining, cells were incubated for 1 h with the combination of rabbit polyclonal anti-(GAP-43) antibody (diluted 1:200 in PBS) and mouse monoclonal anti-(SNAP-25) antibody (diluted 1:100 in PBS) or mouse mono-

clonal anti-syntaxin antibody (diluted 1:100 in PBS). After being washed, the cells were stained with rhodamine-conjugated goat anti-(rabbit IgG) for GAP-43, and FluoroLink Cy2-labelled donkey anti-(mouse IgG) for SNAP-25 or syntaxin.

Protein phosphorylation

For analysis *in vivo*, brain slices (100 mg wet weight of a whole brain) were incubated in 2 ml of phosphate-free Dulbecco's modified Eagle's medium containing 500 μCi of [^{32}P]P_i per tube for 5 h at 37 °C in an air/CO_2 (19:1) atmosphere. After the addition of 1 μM PMA or 0.1 mM H-7, the samples were further incubated for 30 min. The slices were washed twice with ice-cold buffer A and homogenized in 1 ml of buffer A containing 100 mM NaF, 5 mM EDTA and 5 mM EGTA. After centrifugation at 1000 ***g*** for 5 min, aliquots (1/10) of the supernatant (100 μg of protein in 100 μl) were dissolved in SDS/PAGE sample buffer containing 5% (v/v) 2-mercaptoethanol. The remainder of each sample was adjusted to contain 1% (v/v) Triton X-100, 0.5% deoxycholate and 0.1% SDS in a final volume at 0.5 ml, and subjected to immunoprecipitation with anti-(GAP-43) antibody. For analysis *in vitro*, brain slices were incubated in 2 ml of buffer A containing 2.5 mM Ca^{2+} for 30 min at 37 °C, then homogenized as above. Equal amounts of the homogenates (100 μg of protein in 100 μl per tube) were incubated at 37 °C for 5 min with 15 μg/ml digitonin in buffer A. The phosphorylation reaction was started by the addition of 0.2 mM $CaCl_2$ and 100 μCi of [γ-^{32}P]ATP (final concentration 15 μM) in the absence or presence of 1 μM PMA or 0.1 mM H-7. After incubation for 10 min at 30 °C the reaction was stopped by adding 100 μl of buffer A containing 100 mM NaF, 5 mM EDTA and 5 mM EGTA. Each sample was subjected to cross-linking followed by solubilization and immunoprecipitation. ^{32}P-labelled samples were analysed by SDS/PAGE [10% (w/v) gel] followed by autoradiography.

RESULTS

Co-localization of GAP-43 with SNAP-25 and syntaxin

Phaeochromocytoma-derived PC12 cells are differentiated to extend neurites in the presence of NGF, and express SNAP-25, syntaxin and VAMP (synaptobrevin) [33]. The localization of GAP-43, SNAP-25 and syntaxin was examined by double-immunofluorescence staining of the cells (Figure 1). GAP-43 was localized mainly at the growth cones in the NGF-treated cells, where SNAP-25 and syntaxin were co-localized. Synaptotagmin was also detected in the growth cones (results not shown).

GAP-43 interacts with SNAP-25 in NGF-treated PC12 cells

Treatment of PC12 cells with NGF caused no significant change in expression levels of GAP-43 and SNAP-25, compared with those in control cells (Figure 2, lanes 1 and 2). A possible interaction of GAP-43 with SNAP-25 was examined under various conditions. Among several chemical cross-linkers tested, the homobifunctional reagent DSP was found to be most suitable for cross-linking proteins in our system because it is membrane-permeable and thiol-cleavable. We performed the cross-linking with a final concentration of 0.1 mM DSP because higher concentrations resulted in an increase in the formation of large aggregates that could not be solubilized in lysis buffer and were not separated by SDS/PAGE. In the control cells without NGF treatment, GAP-43 was found not to be associated with SNAP-25 even after cross-linking of proteins with DSP (Figure 2, lanes 3 and 4). In contrast, the interaction of GAP-43 with SNAP-25 was detected in DSP-treated homogenates prepared from the NGF-treated cells (Figure 2, lane 6). However, this interaction was not detected in a sample for which cells were solubilized with 1% Triton X-100 before DSP treatment (Figure 2, lane 8), as well as in samples without DSP treatment (lanes 5 and 7).

The effect of detergents on interaction of the two proteins was then examined (Figure 3), demonstrating that the amount of SNAP-25 cross-linked with GAP-43 was greatly decreased by pretreatment of the cell homogenates with the indicated detergents, except for 0.5% n-octylglucoside (Figure 3, lane 8). However, the interaction was not affected by the presence of 0.5 M NaCl (lane 2). Taken together, these results indicate that GAP-43 is associated with SNAP-25 only in the cells differentiated by NGF and that their interaction can be efficiently detected if the membrane remains intact.

Interaction of GAP-43 with the synaptic core complex

GAP-43 is known to be attached to the presynaptic membrane by two acylated Cys residues near the N-terminus [34]. The interaction of GAP-43 with SNAP-25, another acylated protein, was confirmed to occur in the membrane (Figure 4, lanes 1–4). In addition it was found that GAP-43 was also associated with the other synaptic core complex components syntaxin and VAMP, when analysed under the same conditions (Figure 4, lanes 5–7), suggesting that all these proteins are associated with each other in the membrane. However, the nature of the interaction between GAP-43 and the core complex including SNAP-25, syntaxin and VAMP is clearly different from that of those components in the core complex that are stably associated even in the presence of the detergent SDS [35].

GAP-43 and calmodulin dissociate from the core complex by Ca^{2+} influx in brain tissues

The final step in the exocytosis of synaptic vesicles is triggered by Ca^{2+} influx [36]. The molecular mechanism underlying the event has not yet been elucidated. To approach this issue we examined the effect of a Ca^{2+} influx on the interaction of GAP-43 with calmodulin, the core complex and synaptotagmin in rat brain tissue. When brain slices were incubated in the absence of Ca^{2+} (chelated with 5 mM EGTA) and subjected to immunoprecipitation with anti-(GAP-43), GAP-43 was found to maintain an interaction with all the other components (Figure 5A, lane 3). This might reflect a cellular situation without a new entry of Ca^{2+} under which fusion and recycling of vesicles already docked to the plasma membrane are blocked. In contrast, GAP-43 was dissociated from these proteins in the slices incubated with 2.5 mM Ca^{2+} (Figure 5A, lane 4), possibly owing to the fusion of already docked vesicles occurring in response to Ca^{2+} influx. Similar results were obtained by analysis of samples immunoprecipitated with anti-calmodulin (Figure 5B), suggesting that calmodulin is associated with the core complex via GAP-43, which contains a calmodulin-binding domain [14,15,37].

Effect of Ca^{2+} influx on the interaction of core complex components

Although these interactions of GAP-43 were detectable only after being cross-linked with DSP (Figures 5A and 5B), interactions of SNAP-25, syntaxin and VAMP were detected without cross-linking (Figure 6), which is consistent with the evidence that the core complex formed by these proteins is tight, even resistant to SDS *in vitro* [35]. The association of synaptotagmin with the core complex components was also detectable only after cross-linking. This result might reflect synaptotagmin's putative

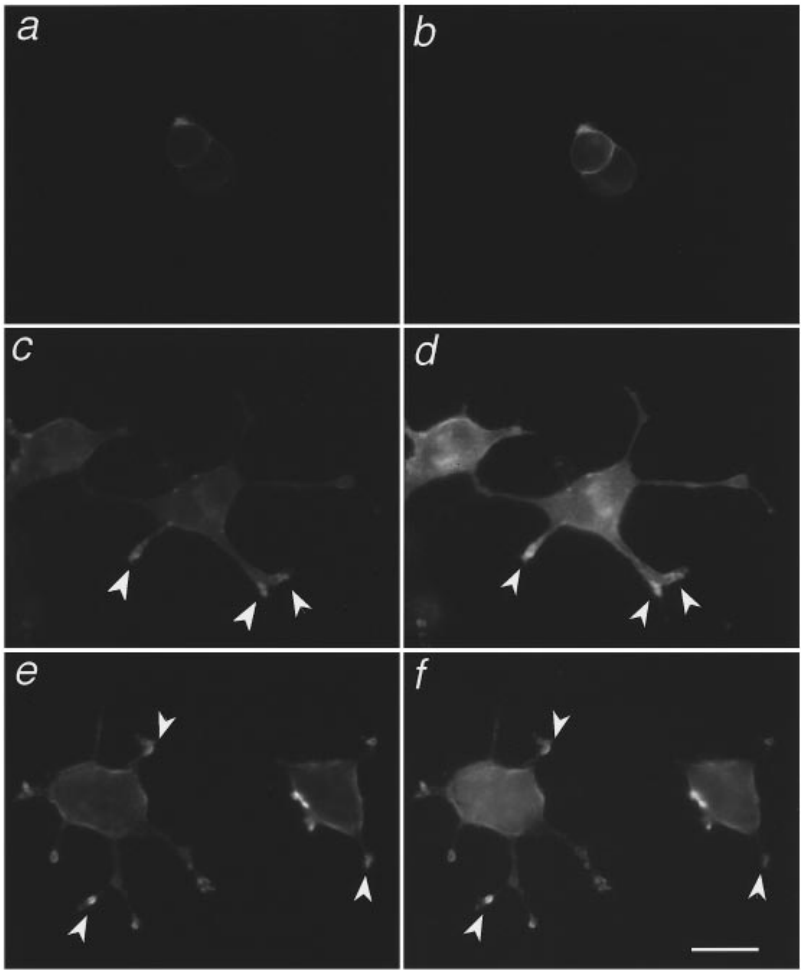

Figure 1 Co-localization of GAP-43 with SNAP-25 and syntaxin in PC12 cells

PC12 cells cultured in the absence (**a**, **b**) or presence (**c**–**f**) of NGF were double-stained by immunofluorescence with antibodies against GAP-43 (**a**, **c**, **e**) and SNAP-25 (**b**, **d**) or syntaxin (**f**) as described in the Materials and methods section. Arrowheads indicate co-localization of GAP-43 with SNAP-25 (**c**, **d**) and syntaxin (**e**, **f**) at the ends of neurites. Scale bar, 10 μm.

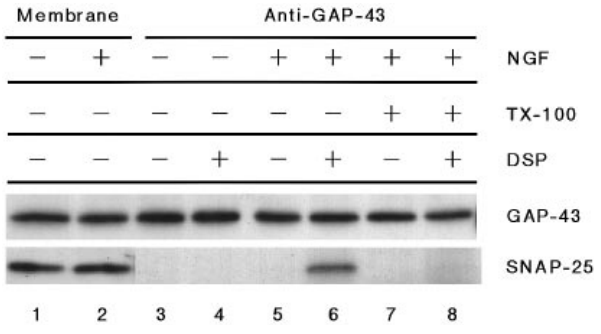

Figure 2 Cell differentiation-dependent interaction of GAP-43 with SNAP-25

Membranes prepared from PC12 cells (1.5×10^6) with (lanes 2 and 5–8) or without (lanes 1, 3 and 4) NGF treatment were analysed by immunoblotting with rabbit anti-(GAP-43) (1/200 of original serum) or mouse anti-SNAP-25 (1/200 of 1 mg/ml IgG) directly (lanes 1 and 2; 10 μg of protein) or after immunoprecipitation with anti-(GAP-43) (lanes 3–8). Anti-(rabbit IgG) or anti-(mouse IgG) was used as secondary antibody. Samples in lanes 4, 6 and 8 were treated with DSP before (lanes 4 and 6) or after (lane 8) being solubilized with 1% Triton X-100, and then subjected to immunoprecipitation after solubilization.

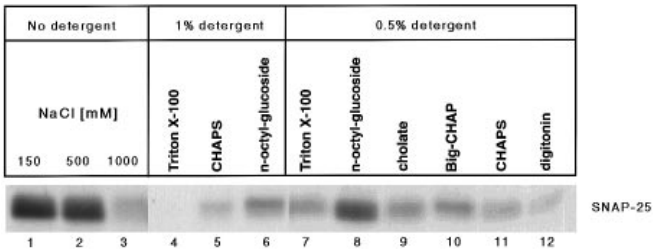

Figure 3 Effects of detergents on the interaction of GAP-43 with SNAP-25

NGF-treated cells (1.5×10^6) were homogenized in 0.2 ml of buffer A containing NaCl but no detergent (lanes 1–3) or lysed with the indicated detergents in buffer A (lanes 4–12). After being treated with DSP, the samples were subjected to immunoprecipitation with rabbit anti-(GAP-43), followed by immunoblotting with mouse anti-(SNAP-25). Anti-(mouse IgG) was used as secondary antibody. The samples in lanes 1–3 were solubilized with 1% Triton X-100 before immunoprecipitation.

association with syntaxin after the core complex has been activated or primed by ATP hydrolysis *in vitro* [22]. In the presence of Ca^{2+}, syntaxin was substantially dissociated from

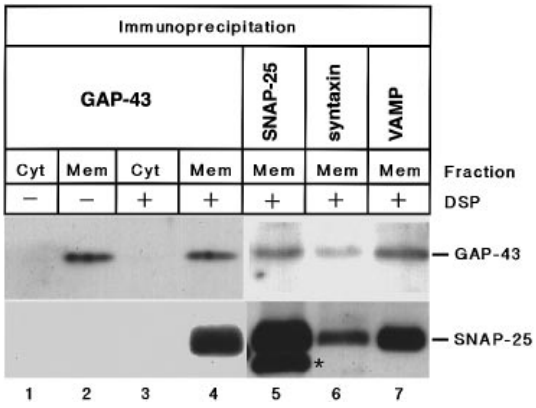

Figure 4 Interaction of GAP-43 with the synaptic core complex in membranes

Homogenates (0.2 ml) from NGF-treated cells (1.5×10^6) were incubated with or without DSP and separated into cytosol (Cyt) and membrane (Mem) fractions by centrifugation. The samples were subjected to immunoprecipitation with rabbit anti-(GAP-43) and anti-VAMP or mouse anti-(SNAP-25) and anti-syntaxin, followed by immunoblotting with rabbit anti-(GAP-43) or mouse anti-(SNAP-25), for which sheep anti-(rabbit IgG) or anti-(mouse IgG) respectively were used as secondary antibodies. The asterisk in lane 5 indicates the light chain of IgG used for immunoprecipitation. Although the light chain of anti-syntaxin IgG was also detected by anti-(mouse IgG), it migrated slightly faster than that of anti-SNAP-25 and is not seen in the trimmed panel (lane 6).

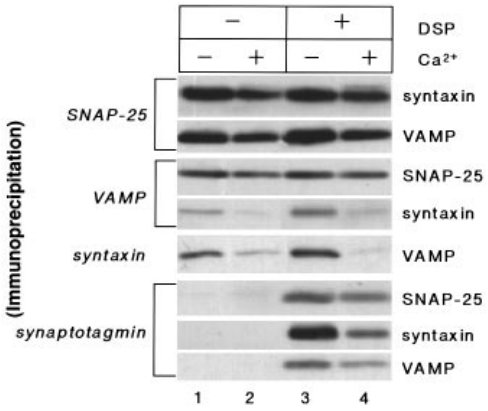

Figure 6 Interaction of the core complex components and synaptotagmin *in vivo*

The lysates of brain homogenates prepared as described in the legend to Figure 5 were first immunoprecipitated with antibodies against the components indicated on the left, and the immunoprecipitates were immunoblotted with antibodies against the components indicated on the right.

VAMP and synaptotagmin (Figure 6 and Table 1), which is consistent with evidence that the core complex is dissociated during the fusion step triggered by Ca^{2+} entry [22,38]. However, the dissociation of SNAP-25 from syntaxin and VAMP was not so prominent, suggesting that there is some difference in association of syntaxin and SNAP-25 with the other components. The lack of the entire dissociation of the core complex might be due to the possibility that each stage during exocytosis was not dissected in this system.

Ca^{2+}-dependence of interactions of the proteins *in vitro*

We then examined interactions of these proteins *in vitro* at various concentrations of Ca^{2+} by using homogenates prepared from brain slices under conditions for dissociation of the proteins. GAP-43 was found to reassociate with SNAP-25, syntaxin and synaptotagmin dependent on concentrations of Ca^{2+}, reaching maximum binding at 100 μM Ca^{2+} (Figures 7A and 7B). A similar Ca^{2+}-dependent interaction was observed between calmodulin and the other components (Figures 7C and 7D). Synaptotagmin also associated with syntaxin in a Ca^{2+}-dependent manner, with maximum binding at 200 μM Ca^{2+} ($n = 3$) (Figures 8A and 8B). These results indicate that GAP-43 and calmodulin interact with the core complex in a Ca^{2+}-dependent manner, suggesting that GAP-43 as well as calmodulin might contribute to Ca^{2+}-triggered membrane fusion by collaborating with synaptotagmin, which is believed to function as a Ca^{2+} sensor [27,39].

The interactions of the other components including SNAP-25/VAMP, syntaxin/VAMP, and synaptotagmin/SNAP-25 and

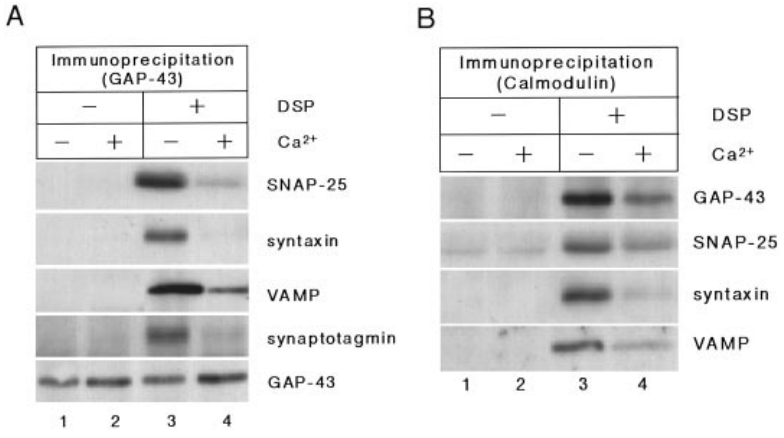

Figure 5 Interaction of GAP-43 and calmodulin *in vivo* with core complex and related components in rat brain

(**A**) Brain slices (100 mg wet weight) were incubated at 37 °C for 30 min in buffer A in the presence of 5 mM EGTA ($-Ca^{2+}$) or 2.5 mM Ca^{2+} ($+Ca^{2+}$) as described the Materials and methods section. The slices were immediately homogenized in the same buffer and centrifuged. Equal amounts of the homogenates (100 μg of protein in 100 μl) before or after cross-linking were lysed and subjected to immunoprecipitation with anti-(GAP-43) antibodies (30 μg of IgG). The immunoprecipitates were analysed by immunoblotting with antibodies against the indicated components. (**B**) Immunoprecipitates obtained from the homogenates with anti-calmodulin antibodies were analysed by immunoblotting with antibodies against the indicated components, as described in (**A**).

Table 1 Effect of Ca^{2+} influx on the interaction of the core complex components and synaptotagmin in brain tissues

Data shown in Figure 6 were quantified by the NIH image program as described in the Materials and methods section. Values obtained in the presence of Ca^{2+} for each component are expressed as percentages of those obtained in the absence of Ca^{2+} (% of $-Ca^{2+}$) and are the means ± S.D. for three separate experiments. Abbreviation: n.d., difference not detectable.

Component immunoprecipitated	Component immunoblotted	Interaction (% of $-Ca^{2+}$)	
		Without DSP	With DSP
SNAP-25	Syntaxin	53.4 ± 7.1	73.9 ± 3.5
	VAMP	53.6 ± 6.8	38.7 ± 4.4
VAMP	SNAP-25	46.1 ± 2.6	51.3 ± 5.8
	Syntaxin	15.0 ± 3.5	15.5 ± 4.9
Syntaxin	VAMP	22.1 ± 3.8	9.8 ± 2.4
Synaptotagmin	SNAP-25	n.d.	65.2 ± 5.5
	Syntaxin	n.d.	30.6 ± 4.5
	VAMP	n.d.	45.1 ± 3.7

VAMP occurred independently of Ca^{2+} concentration and even in the presence of EGTA (Figures 8A and 8B), possibly because of reassociation during post-incubation even in the absence of Ca^{2+}.

Effect of GAP-43 phosphorylation on the interaction between GAP-43 and the core complex

GAP-43 is a well-known PKC substrate in neurons [8,9]. It was reported that the phosphorylation of GAP-43 by PKC was coupled with neurotransmitter release by using synaptosomes [10,40,41]. In fact, treatment of brain slices with PMA, which is a potent PKC activator, increased the phosphorylation of GAP-43 more than 7-fold compared with that in control cells and in cells treated with H-7, which is a known PKC inhibitor (Figure 9A). To examine whether the phosphorylation of GAP-43 could affect the interaction with the core complex, homogenates were prepared from slices that had been preincubated in the presence

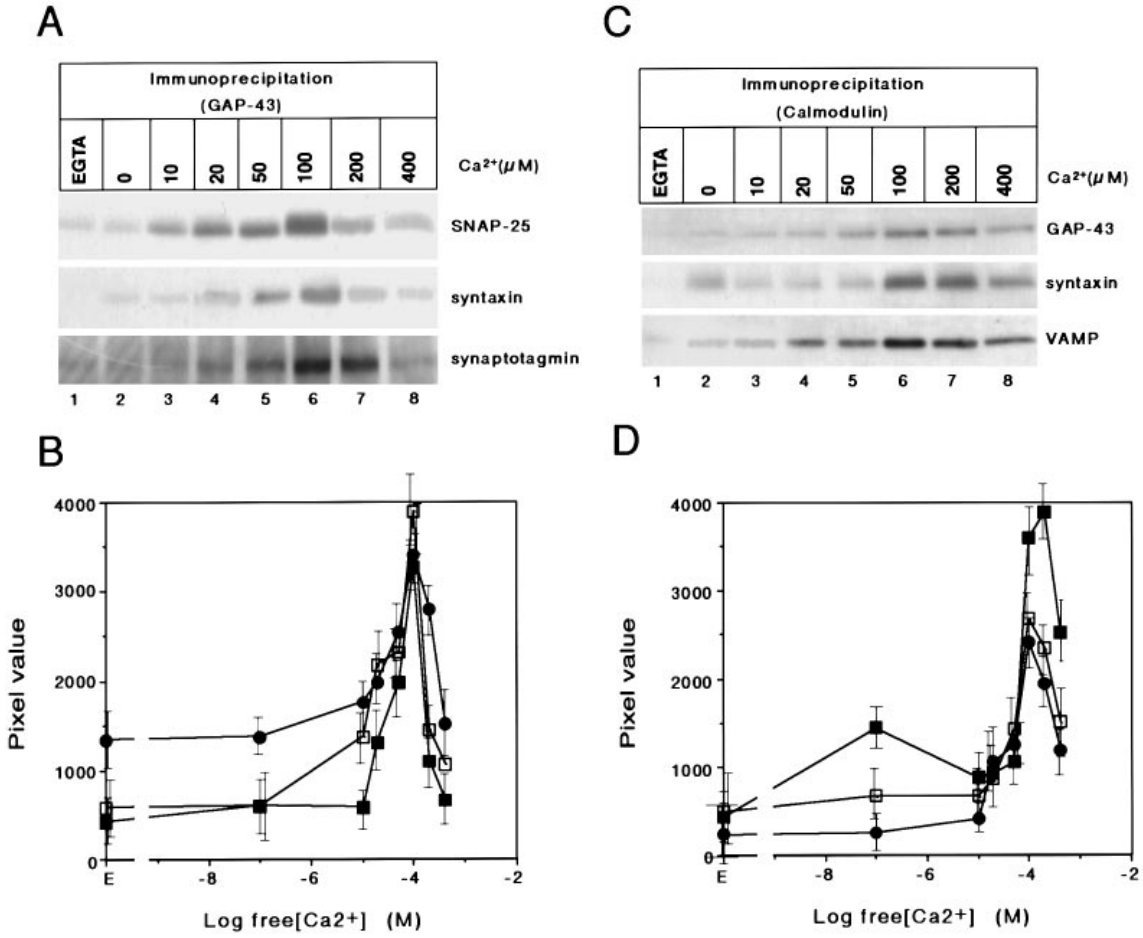

Figure 7 Ca^{2+}-dependence of interactions of GAP-43 and calmodulin with other components *in vitro*

Homogenates were prepared from brain slices incubated in the presence of 2.5 mM Ca^{2+}, as described in the legend to Figure 5. Equal amounts of the homogenates (100 μg of protein in 100 μl) were incubated at 37 °C for 30 min in buffer A with the indicated concentrations of Ca^{2+} or 5 mM EGTA. Each sample was cross-linked, solubilized and immunoprecipitated for GAP-43 (**A**) and calmodulin (**C**) followed by immunoblotting with antibodies against the proteins indicated on the right of each panel. (**B**, **D**) Quantitative analysis of the immunoblotting data in (**A**) and (**C**) respectively, performed as described in the Materials and methods section. The pixel values obtained were plotted along the corresponding points of free [Ca^{2+}]. Results are means ± S.D. for three independent experiments. In (**B**), SNAP-25 (□), syntaxin (■) and synaptotagmin (●) were immunoprecipitated with anti-(GAP-43). In (**D**), GAP-43 (□), syntaxin (■) and VAMP (●) were immunoprecipitated with anti-calmodulin. Note that Ca^{2+} contamination at the zero point was estimated to be 100 nM. Abbreviation: E, the point of addition of EGTA.

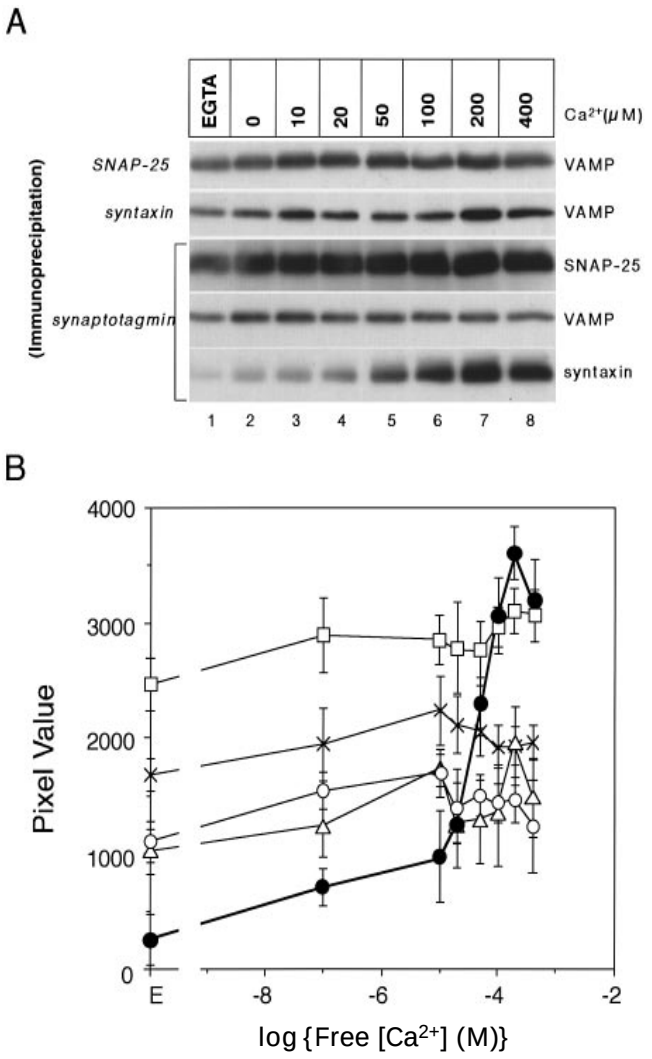

Figure 8 Interactions of the core complex components *in vitro*

(**A**) The lysates were prepared after the homogenates had been treated under the same conditions as described in the legend to Figure 7. The lysates were first immunoprecipitated with antibodies against the components indicated on the left, and the immunoprecipitates were then immunoblotted with antibodies against the components indicated on the right. (**B**) Quantitative analysis of data in (**A**) was performed under the same conditions as described in the legend to Figure 7. Results are means ± S.D. for three independent experiments. The combinations of immunoblotting/immunoprecipitation are as follows; VAMP/SNAP-25 (×), VAMP/syntaxin (△), SNAP-25/synaptotagmin (□), VAMP/synaptotagmin (○), syntaxin/synaptotagmin (●).

of 2.5 mM Ca^{2+} in the absence or presence of PMA or H-7 and cross-linked with DSP, followed by immunoprecipitation with anti-syntaxin antibody. The intensity of ^{32}P-labelled GAP-43 detected in the PMA-treated sample was much heavier than that in the other samples (Figures 9B and 9C). To compare the interaction of GAP-43 with the core complex under these conditions, immunoprecipitates obtained with anti-SNAP-25, anti-syntaxin and anti-GAP-43 were subjected to immunoblotting for GAP-43. As shown in Figures 9(D) and 9(E), the interaction of GAP-43 with SNAP-25 and syntaxin was markedly increased in the presence of PMA. These results suggest that the phosphorylation of GAP-43, possibly by PKC, enhances the interaction with the synaptic core complex.

DISCUSSION

Because it is expressed at a high level during the axonal growth and regeneration, GAP-43 has been proposed to be a determinant for neurite outgrowth [30,42,43] and plasticity [44,45]. However, recent studies on neuronal cells with no expression of GAP-43 show that the protein is not essential for neurite formation or extension. A PC12 cell line deficient in GAP-43 was capable of NGF-, basic fibroblast growth factor- and cAMP-mediated neurite outgrowth [46]. The formation of both growth cones and neurites also occurred in chick sensory neurons depleted of GAP-43 by anti-sense cRNA expression and in cells from GAP-43-deficient mice created by homologous recombination [47,48]. Instead it was observed that the growth cones failed to produce NGF-induced spreading or insulin-like growth factor 1-induced branching in the deficient cells.

The predominant localization of GAP-43 on the cytoplasmic side of the presynaptic plasma membrane [1] raises the possibility that GAP-43 might play a role in the formation of a specialized area in the presynaptic membrane (the active zone) where the exocytosis of synaptic vesicles occurs [20]. In fact the present study demonstrated the interaction of GAP-43 with the core complex components that are required for docking/fusion of synaptic vesicles to the plasma membrane. The interaction was detected in rat brain tissues and PC12 cells differentiated by NGF but not in undifferentiated cells despite their expressing the proteins at the same levels, suggesting a functional role of the interaction in differentiated neural cells. This result might correlate with previous morphological studies that the expression of GAP-43 during NGF-induced differentiation in PC12 cells is accompanied by a shift in its localization from lysosomal structures and Golgi apparatus to the plasma membrane [49]. Furthermore there is a close correlation between the accumulation of GAP-43 in growth cones and the expression of neuronal polarity in hippocampal neurons in primary culture [43]. In addition to a determinant for active zone formation, GAP-43 might play a role in rapid neurotransmitter release, for example, by increasing the number of docked vesicles at the release site and by regulating the Ca^{2+}-triggering step. In contrast with the synapse, exocytosis in other regulatory secretory cells, such as adrenal chromaffin cells, pituitary cells and mast cells, is triggered more slowly [50,51] because the secretory granules are held by an actin network [52]. Recently it was observed that the expression of GAP-43 in mouse anterior pituitary AtT-20 cells facilitates the K^+-evoked secretion of endorphin [53]. It will be very interesting to study whether GAP-43 interacts with the SNARE components in this cell. Our results from PC12 cells might be closely related to the specificity of neuronal exocytosis as distinct from other regulated secretion.

The interactions of GAP-43 with the core complex components calmodulin and synaptotagmin could be detected only when chemical cross-linking of proteins was performed before they were solubilized from the membrane with detergents. This might be why the findings presented here have not been observed by other investigators, who used Triton X-100 extracts of bovine brain membranes [22,54]. GAP-43 is attached to the presynaptic membrane by fatty acylation of two Cys residues at positions 3 and 4 [34]. It is suggested by mutational analysis that the N-terminal 20 amino acid residues containing the two Cys and adjacent basic residues are essential for targeting GAP-43 to the growth cones or presynaptic membranes [55,56]. At present it is not known how this occurs, or which domain of GAP-43 interacts with these components. The interaction is, however, considered to be specific because it is observed only in differentiated PC12 cells and maintained in the presence of high salt concentrations

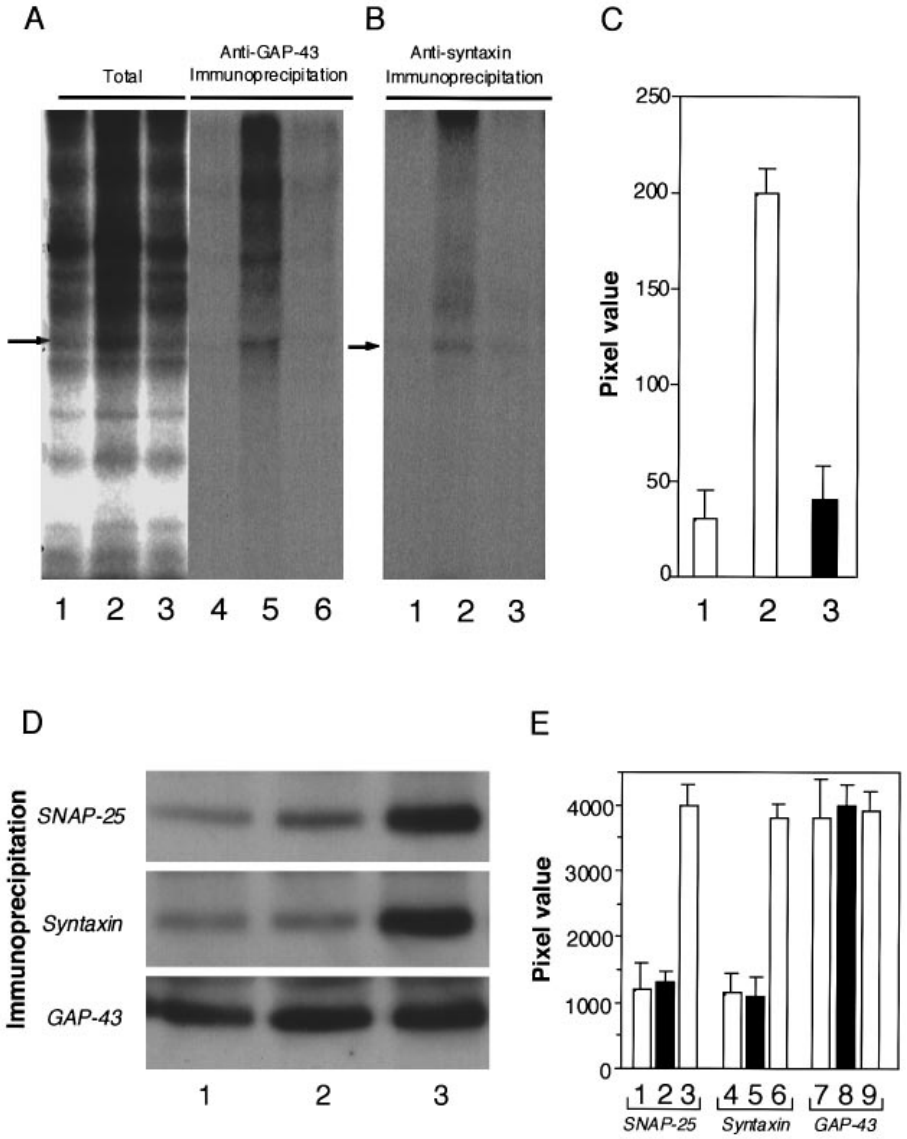

Figure 9 Effect of PKC-activated phosphorylation on the interaction between GAP-43 and the core complex in rat brain

(**A**) Brain slices (100 mg wet weight) were labelled with [^{32}P]P_i in the absence (lanes 1 and 4) or presence of 1 μM PMA (lanes 2 and 5) or 0.1 mM H-7 (lanes 3 and 6). The slices were homogenized, and GAP-43 was immunoprecipitated as described in the Materials and methods section. Total homogenates (lanes 1–3) and the immunoprecipitates (lanes 4–6) were analysed by SDS/PAGE [10% (w/v) gel] followed by autoradiography. The position of GAP-43 is indicated by an arrow. (**B**) Homogenates were prepared from brain slices incubated in the presence of 2.5 mM Ca^{2+} as described in the legend to Figure 5. Equal amounts of the homogenates (100 μg of protein in 100 μl) were preincubated in 15 μg/ml digitonin and phosphorylated with [γ-^{32}P]ATP in the absence (lane 1) or presence of 1 μM PMA (lane 2) or 0.1 mM H-7 (lane 3). Each sample was cross-linked, solubilized and immunoprecipitated with anti-syntaxin antibody, and then analysed by SDS/PAGE [10% (w/v) gel]/autoradiography. (**C**) The bands of GAP-43 shown in (**B**) were quantified by the NIH image program as described in the Materials and methods section. Results are expressed as the pixel values of the means $\pm$ S.D. for three separate experiments in the absence (bar 1) or presence of 1 μM PMA (bar 2) or 0.1 mM H-7 (bar 3). (**D**) Homogenates were prepared under the same conditions as described for (**B**) except that there was no addition of [γ-^{32}P]ATP, in the absence (lane 1) or presence of 0.1 mM H-7 (lane 2) or 1 μM PMA (lane 3). Immunoprecipitates were obtained from the homogenates with the antibodies indicated at the left, and subjected to immunoblotting with anti-(GAP-43). (**E**) Quantitative analysis of data in (**D**) was performed under the same conditions as described in the legend to Figure 7. The Figure shows the means $\pm$ S.D. for triplicate experiments in the absence (bars 1, 4 and 7) or presence of 0.1 mM H-7 (bars 2, 5 and 8) or 1 μM PMA (bars 3, 6 and 9).

(0.5 M NaCl). The conditions required for detection of the interactions suggest that the interaction of GAP-43 with the other components is maintained only on the intact membrane but is easily disrupted when the membrane topology of each component is perturbed with the detergents.

Our results obtained by the experiments *in vitro* indicate that GAP-43 and calmodulin associate with the core complex with a maximum binding affinity at 100 μM Ca^{2+}. In addition, the interaction between syntaxin and synaptotagmin shows the maximum affinity to be at 200 μM Ca^{2+}, as reported previously [39,57]. These Ca^{2+} concentrations are close to that required for the Ca^{2+}-dependent release of neurotransmitters [58]. Recently it has been reported that the N-type Ca^{2+} channel interacts with the synaptic core complex in a Ca^{2+}-dependent manner, with a maximum affinity at 20 μM [59], close to the threshold for the initiation of neurotransmitter release. Taken together, these observations suggest that the Ca^{2+}-triggered step in exocytosis of synaptic vesicles might proceed with the sequential interactions

of the N-type Ca^{2+} channel, GAP-43/calmodulin, and synaptotagmin with the synaptic core complex in response to the Ca^{2+} influx. A working model of synaptic vesicle exocytosis based on findings obtained by experiments *in vitro* indicates that synaptotagmin initially interacts with the SNARE complex (7 S complex) and is then displaced from the complex by αSNAP, resulting in no participation in the formation of the 20 S complex [22,29]. However, considering that synaptotagmin is also suggested to participate as a low-affinity Ca^{2+} sensor in the late step [27], it is reasonable that synaptotagmin would interact again with syntaxin and/or other components in the final fusion step [28,57]. It has been suggested by other approaches that GAP-43 is involved in the mechanism of neurotransmitter release [16–19]. Our results support the notion more directly at the molecular level by demonstrating the interaction of GAP-43 with the components involved in the exocytosis.

The fact that calmodulin also interacts with GAP-43 and/or the core complex suggests that calmodulin has an important role in the Ca^{2+}-triggered fusion step. The interaction might regulate Ca^{2+}/calmodulin-dependent protein kinase II, which is implicated in the regulated release of neurotransmitters such as glutamate and noradrenaline, and by the phosphorylation of synapsin in the synaptic vesicles [60,61]. Finally, the amount of GAP-43 associated with the synaptic core complex was enhanced by PKC activation *in vitro*. It remains to be determined whether the PKC-mediated phosphorylation of GAP-43 regulates the docking or fusion of the synaptic vesicles *in vivo*. The functional role of phosphorylation of GAP-43/calmodulin in exocytosis will be clarified by the construction of mutants without phosphorylation sites and an analysis of their interaction with the core complex in cells expressing the mutants. An investigation along this line is now in progress in our laboratory.

We thank Dr. A. Uehara and Dr. T. Fujiwara (Fukuoka University, Fukuoka, Japan) for useful advice, and Dr. S. Hori and Dr. T. Mori (Oita Medical School, Oita, Japan) for encouragement. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and by the Science Research Promotion Fund from the Japan Private School Promotion Foundation.

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Received 21 November 1996/24 February 1997; accepted 18 March 1997