Synaptophysin is a synaptic vesicle (SV) protein of unknown function. Here we show that a repeated sequence in the cytoplasmic tail of synaptophysin mediates the formation of a protein complex containing the GTPase dynamin. The formation of this complex requires a high Ca\(^{2+}\) concentration, suggesting that it occurs preferentially at the sites of SV exocytosis. Coimmunoprecipitation of a dynamin-synaptophysin complex from brain extracts is promoted by dissociation of vesicle-associated membrane protein 2 from synaptophysin. This finding suggests that dynamin only associates with synaptophysin in vivo after vesicle-associated membrane protein 2 (VAMP2) enters the SNARE complex. GTP binding releases dynamin from synaptophysin, possibly serving to regulate dynamin self-assembly during endocytosis. Our results suggest that synaptophysin plays a role in SV recycling by recruiting dynamin to the vesicle membrane.

Synaptophysin is one member of a family of SV proteins with four transmembrane domains (1, 2). In addition to synaptophysin, SVs contain the closely related protein synaptoporin (3, 4) and the distantly related protein synaptogyrin (5). The function of these proteins is currently unknown. Synaptic transmission, synaptic plasticity, and SV number are unaffected in synaptophysin knockout mice (6, 7), although synaptophysin-synaptogyrin double knockout mice exhibit defects in synaptic plasticity (8). The molecular basis for these defects is unknown.

The only protein known to interact directly with synaptophysin is vesicle-associated membrane protein 2 (VAMP2) (9, 10), which mediates vesicle fusion via SNARE complex formation with syntaxin and SNAP 25. Synaptophysin-VAMP 2 complexes purified from synaptosomal extracts do not contain syntaxin 1 or SNAP 25, suggesting that VAMP 2 dissociates from synaptophysin prior to entering the SNARE complex (10). These data raise the possibility that the association of VAMP 2 with synaptophysin serves to regulate the availability of VAMP 2 for SNARE complex formation. This hypothesis is supported by a study demonstrating an inverse correlation between the basal rate of SV exocytosis and the extent of synaptophysin-VAMP 2 association (11).

Synaptophysin has also been shown to bind cholesterol, which appears to be required for SV formation (12). This observation suggests a function for synaptophysin in the biogenesis of SVs, a role that had been proposed previously based on the ability of synaptophysin to induce the formation of small clear vesicles in non-neuronal cells (13).

To help understand synaptophysin function, we sought to identify synaptophysin binding partners. In this report, we describe a Ca\(^{2+}\)-dependent interaction between synaptophysin and dynamin I, a GTPase required for SV endocytosis (14, 15). Our results suggest that synaptophysin may function in vesicle recycling. Indeed, this hypothesis is supported by recent in vivo experiments performed in the squid giant synapse (16).

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Fragments of the synaptophysin cDNA (17) (a gift of T. Sudhof) encoding various regions of the synaptophysin C terminus were amplified by PCR and cloned into the BamHI-SalI sites of the pGEX-4T-1 vector (Amersham Biosciences, Inc.), creating plasmids that encode GST-SYP fusion proteins. Fragments of the synaptophysin II (3) and synaptogyrin (5) cDNAs encoding their C-terminal cytoplasmic domains (amino acids 199–265 of synaptophysin II and amino acids 170–234 of synaptogyrin) and a fragment of the amphipathic II cDNA encoding amino acids 491–588 of the SH3 domain were amplified from a rat brain cDNA library by PCR and cloned into pGEX-4T-1.

**Binding Assays Using Immobilized GST Fusion Proteins**—The various GST fusion proteins were expressed in *Escherichia coli* and purified on glutathione-agarose beads. A washed synaptosome fraction was prepared from the cerebral cortex of 6–8-week-old Sprague-Dawley rats essentially as described previously (18). Synaptosomes were extracted in 50 mM NaCl, 20 mM HEPES, pH 7.4, 1% Triton X-100, and a protease inhibitor mixture (Complete™ protease inhibitor without EDTA, Roche Molecular Biochemicals) for 1 h at 4 °C and then clarified by centrifugation at 15,000 × g for 10 min. Synaptosomal extracts at a protein concentration of ~7.5 mg/ml (unless stated otherwise) were incubated in a volume of 1 ml with ~50 μg of the indicated GST fusion proteins immobilized on 20 μl of glutathione-agarose beads (Santa Cruz Biotechnology, Inc.) for 2–3 h at 4 °C. Binding reactions generally contained either 2 mM EGTA or CaCl\(_2\) unless noted otherwise. After binding, the beads were washed three times with 1 ml of extraction buffer containing EGTA or Ca\(^{2+}\). Bound proteins were eluted by boiling in SDS gel sample buffer and resolved on an SDS gel.

**Effect of GTP on Dynamin-Synaptophysin Complex**—To test the effect of nucleotides on the ability of dynamin to bind to synaptophysin (Fig. 5A), synaptosomal extracts were incubated with GST-SYP in the presence of Ca\(^{2+}\) and either no nucleotide or 10 μM GDP or 100 μM GTP·S. All samples contained 1 mM Mg\(^{2+}\). To test whether GTP could release dynamin from synaptophysin (Fig. 5B), synaptosomal extracts were incubated with GST-SYP in the presence of Ca\(^{2+}\). The beads containing the dynamin-synaptophysin complexes were then washed three times with buffer containing no nucleotide or 10 μM GDP, 100 μM GTP, or 100 μM GTP·S. All wash buffers contained 1 mM Mg\(^{2+}\). After washing, the amount of dynamin remaining bound to synaptophysin was assayed by immunoblot.
**RESULTS**

**Ca^{2+}-dependent Association of Dynamin with the Synaptophysin-Cytoplasmic Tail**—To investigate whether synaptophysin associates with synaptic proteins other than VAMP 2, a fusion protein of GST and the 89 amino acid C terminus of synaptophysin (GST-SYP) was immobilized on beads and incubated with a synaptosomal extract. Because Ca^{2+} is known to modulate many protein-protein interactions in synapses, the binding was done in the presence of EGTA or Ca^{2+}. Synaptosomal proteins that bound to GST or GST-SYP were eluted, resolved on an SDS gel, and visualized by Coomassie Blue staining. In the presence of Ca^{2+} but not EGTA, GST-SYP bound to a protein of approximately 100 kDa (Fig. 1B). This protein, which was not bound by GST in the presence of either EGTA or Ca^{2+} (Fig. 1B), was subsequently identified by immunoblot as dynamin I, a neuron-specific GTPase required for vesicle recycling. Because dynamin has been shown to associate with synaptic proteins other than VAMP 2, a monoclonal antibody against dynamin I (D25520, Transduction Laboratories) at 1:500 dilution. The blocking of membranes, washing of membranes, and detection of signals were done as described previously (21).

**Immunoprecipitations**—Synaptosomes were extracted in 0.15 M NaCl, 20 mM HEPES, pH 7.4, 1% Triton X-100, and a protease inhibitor mixture without EDTA for 1 h at 4 °C and then clarified at 15,000 × g for 10 min. Synaptosomal extracts at a protein concentration of ~2.5 mg/ml in a volume of 1 ml were precleared with 15 μl of protein A or protein G-agarose beads (Santa Cruz Biotechnology, Inc.) for 1 h. Extracts were then incubated at 4 or 30 °C for 30 min in the presence of 2 mM EGTA or 2 mM CaCl₂, 2 mM MgCl₂ as indicated in the legend to Fig. 4. The Ca^{2+}/Mg^{2+} treatment served to dissociate synaptophysin from VAMP, the dissociation does not require the addition of ATP (19). Extracts were then placed on ice and immunoprecipitated with 3 μg of a monoclonal antibody against dynamin I (D25520, Transduction Laboratories), 10 μl of a rabbit antiserum against synaptophysin (SY38, Roche Molecular Biochemicals) at 1:1000 dilution (Fig. 4C), or a monoclonal antibody against syntaxin I (HPC-1, Sigma) at 1:1000 dilution (Fig. 4C). Immunoprecipitates were washed three times with 1 ml of extraction buffer containing EGTA or Ca^{2+}. Bound proteins were eluted and resolved on an SDS gel.

**Immunoblot**—The following antibodies were used in immunoblots at the indicated concentrations: monoclonal antibody against dynamin I (D25520, Transduction Laboratories) at 0.5 μg/ml; monoclonal antibody against synaptophysin (SY38, Roche Molecular Biochemicals) at 0.5 μg/ml; monoclonal antibody against syntaxin I (HPC-1, Sigma) at 1:1000 dilution; polyclonal antibody against VAMP2 (SV006, Stressgen Biotechnology), or 1–2 μl (ascites fluid) of a monoclonal antibody against syntaxin I (HPC-1, Sigma). Immunoglobulins were collected with 15 μl of either protein A- or protein G-agarose beads for 1 h at 4 °C. Immunoprecipitates were washed three times with 1 ml of extraction buffer containing EGTA or Ca^{2+}. Bound proteins were eluted and resolved on an SDS gel.

**Fig. 1. Ca^{2+}-dependent binding of dynamin to the C-terminal cytoplasmic tail of synaptophysin. A** topology of synaptophysin in the synaptic vesicle membrane. The arrow indicates the region of the protein (amino acids 219–307), which was fused to GST and used as bait in the binding studies. **B**, the GST-SYP fusion protein and GST alone were immobilized on glutathione-agarose beads and incubated with a synaptosomal extract (protein concentration ~10.5 mg/ml) in the presence of EGTA (**E**) or Ca^{2+} (**Ca**). Bound proteins were eluted by boiling in SDS gel sample buffer, resolved on an SDS gel, and visualized by staining with Coomassie Blue. The arrow indicates a protein of ~100 kDa that was bound specifically by GST-SYP in the presence of Ca^{2+}. C, a synaptophysin extract was incubated with GST or GST-SYP in the presence of EGTA or Ca^{2+}. Bound proteins were eluted, resolved on an SDS gel along with an aliquot of the extract, and subjected to immunoblot with an anti-dynamin antibody (top), an anti-syntaxin 1 antibody (middle), or an anti-synaptotagmin antibody (bottom).
a region of approximately 60 amino acids that is composed almost entirely of the nine YG(P/Q)QG repeats. The precise requirements for the formation of the dynamin-synaptophysin complex will require more extensive mutagenesis of the synaptophysin C terminus. However, this result may explain the ability of synaptophysin II and synaptogyrin to associate with dynamin albeit with much lower affinity (Fig. 3B). Both synaptophysin II and synaptogyrin have sequences that are related to the synaptophysin repeats, for example, YGSSG and YSQQA in synaptophysin II and YQSQG in synaptogyrin (Fig. 3A). 

Association of Dynamin with Synaptophysin Is Promoted by Dissociation of Synaptophysin from VAMP—It has been shown recently that the synaptophysin-VAMP 2 complex can be dissociated in synaptosomal extracts by incubation at 30 °C in the presence of Ca²⁺/Mg²⁺ (19). Although the mechanism underlying this effect is undefined, it seems likely that the addition of Ca²⁺ to the extract activates a process that is normally triggered in vivo by depolarization-induced Ca²⁺ influx (19). A period of strong synaptic activity, by promoting synaptophysin-VAMP 2 dissociation, would thereby replenish the pool of SVs in which VAMP 2 is free to enter the SNARE complex. Because dynamin presumably binds synaptophysin after SNARE complex formation, it seemed possible that the disruption of synaptophysin-VAMP 2 complexes in synaptosomal extracts might release synaptophysin to bind dynamin. The incubation of synaptosomal extracts at 30 °C for 30 min in the presence of Ca²⁺ but not in the presence of EGTA almost completely dissociated synaptophysin from VAMP 2 as assessed by coimmunoprecipitation of synaptophysin with anti-VAMP 2 antibody (Fig. 4A). As shown in Fig. 4B, following incubation of extracts at 30 °C in the presence of Ca²⁺ but not in the presence of EGTA, synaptophysin coimmunoprecipitated with dynamin. Thus, under conditions in which synaptophysin dissociates from VAMP 2, a dynamin-synaptophysin complex forms. Anti-dynamin antibody did not immunoprecipitate syntaxin 1 in the presence of either EGTA or Ca²⁺, and anti-syntaxin 1 antibody did not immunoprecipitate either synap-

**FIG. 2.** Binding of dynamin to synaptophysin requires a Ca²⁺ concentration found only at sites of synaptic vesicle exocytosis. **A**, a synaptosomal extract was incubated with GST-SYP in the presence of EGTA (E) or Ca²⁺ at concentrations of 50, 100, 200, 300, 400, or 500 μM. Bound proteins were eluted, resolved on an SDS gel, and subjected to immunoblot with an anti-dynamin antibody. **B**, the graph illustrates relative dynamin binding to GST-SYP as a function of Ca²⁺ concentration. Data points (% maximal binding ± S.E.) were generated by quantitating signals from immunoblots using NIH Image software.

**FIG. 3.** Dynamin binds to a repeated sequence in the synaptophysin C terminus. **A**, sequences of the cytoplasmic C-terminal domains of synaptophysin (syp), synaptophysin II (syp II), and synaptogyrin (sgyrin). The conserved juxtamembrane and extreme C-terminal sequences of synaptophysins are underlined. The nine pentapeptide repeats in the synaptophysin C terminus with consensus sequence YG(P/Q)QG are boxed. **B**, GST fusion proteins containing the C-terminal cytoplasmic domains of synaptophysin (SYP), synaptophysin II (SYP II), or synaptogyrin (SGYRIN) were immobilized on glutathione-agarose beads and incubated with a synaptosomal extract in the presence of EGTA (E) or Ca²⁺ (Ca). Bound proteins were eluted by boiling in SDS gel sample buffer, resolved on an SDS gel, and subjected to immunoblot with an anti-dynamin antibody. After immunoblotting (IB), the filter was stained with Ponceau S to reveal the GST fusion proteins. **C**, GST fusion proteins containing the indicated regions of the synaptophysin C terminus were immobilized on glutathione-agarose beads and incubated with a synaptosomal extract in the presence of Ca²⁺. Bound proteins were eluted by boiling in SDS gel sample buffer, resolved on an SDS gel, and subjected to immunoblot with an anti-dynamin antibody. After immunoblotting, the filter was stained with Ponceau S to reveal the GST fusion proteins. The sites of the truncations are indicated in A, Δ227–274 is a deletion of the indicated amino acids.
Ca²⁺ and at the same time the amount of synaptophysin coimmunoprecipitated with anti-syntaxin 1 antibody (Fig. 4B). As shown in Fig. 4C, after incubation of extracts at 4°C in the presence of Ca²⁺, the synaptophysin-VAMP 2 complex remained intact, and anti-syntaxin 1 antibody (a syn 1) or anti-dynamin antibody (a dyn 1) bound proteins were eluted, resolved on an SDS gel, and subjected to immunoblot with anti-synaptophysin antibody (top) or anti-VAMP 2 antibody (bottom). C, synaptosomal extracts were preincubated with 15 μl of protein G-agarose for 1 h. Extracts were then incubated at 30°C in the presence of EGTA or Ca²⁺/Mg²⁺ (Ca) for 30 min, placed on ice, and subjected to immunoprecipitation with anti-VAMP 2 antibody. Bound proteins were eluted, resolved on an SDS gel, and subjected to immunoblot with anti-synaptophysin antibody (top) or anti-syntaxin 1 antibody (bottom). Bound proteins were eluted, resolved on an SDS gel, and subjected to immunoblot with anti-synaptophysin antibody (top) or anti-syntaxin 1 antibody (bottom). Bound proteins were eluted, resolved on an SDS gel, and subjected to immunoprecipitation with anti-syntaxin 1 antibody (top) or anti-dynamin antibody (bottom). Bound proteins were eluted, resolved on an SDS gel, and subjected to immunoprecipitation with anti-syntaxin 1 antibody (top) or anti-dynamin antibody (bottom). Bound proteins were eluted, resolved on an SDS gel, and subjected to immunoprecipitation with anti-syntaxin 1 antibody (top) or anti-dynamin antibody (bottom). Bound proteins were eluted, resolved on an SDS gel, and subjected to immunoprecipitation with anti-syntaxin 1 antibody (top) or anti-dynamin antibody (bottom).

**DISCUSSION**

The results presented in this report indicate that dynamin interacts with synaptophysin in a Ca²⁺-dependent fashion. In addition, we show that the interaction is modulated by dissociation of synaptophysin from VAMP and by GTP binding to dynamin. Given the well established function of dynamin in endocytosis (14, 15), it seems most probable that the association of dynamin with synaptophysin plays a role in vesicle recycling by targeting dynamin to the SV membrane. Indeed, we have recently shown that injection of the dynamin C terminus into the squid giant synapse results in a block of SV release (16). The results presented here suggest that the recycling block results from a disruption of the dynamin-synaptophysin complex.

Several important questions regarding the role of a dynamin-synaptophysin complex in SV recycling remain to be addressed. First, do dynamin and synaptophysin interact directly, or does the association require additional synaptic proteins? Attempts to reconstitute a Ca²⁺-dependent interaction between GST-SYP and dynamin purified from rat brain have yielded inconclusive results (data not shown). One possible explanation for this is that a required protein is missing from the binding reaction. Coomassie Blue staining of the synaptic proteins which bind to the synaptophysin C terminus reveals that dynamin is the major protein (Fig. 1B). However, another protein(s) essential for the interaction might be present in very...
Fig. 5. GTP binding releases dynamin from synaptophysin. A, GST-SYP or GST-Amph SH3 were immobilized on glutathione-agarose beads and incubated with a synaptosomal extract. The binding reactions were supplemented with Mg\(^{2+}\) (1 mM) and GDP (10 \(\mu\)M) or GTP, as indicated. Bound proteins were eluted, resolved on an SDS gel, and subjected to immunoblot with anti-dynamin antibody. B, dynamin-synaptophysin or dynamin-amphiphysin complexes were formed by incubating synaptosomal extracts with GST-SYP in the presence of Ca\(^{2+}\) (left) or with GST-Amph SH3 (right). Half of each sample was then processed for immunoblotting as usual to confirm that approximately equal levels of dynamin binding had occurred (top). The rest of each sample was washed with buffer containing Mg\(^{2+}\) (1 mM) and GDP (10 \(\mu\)M), GTP (100 \(\mu\)M), or GTP-S\(^{\gamma}\)S (100 \(\mu\)M), as indicated. The dynamin, which remained bound after washing, was eluted, resolved on an SDS gel, and subjected to immunoblot with anti-dynamin antibody (bottom).
plasmids, and A. Bhamidipati, P. De Camilli, A. Petrenko, and P. Robinson for helpful discussions.

Note Added in Proof—While this manuscript was under review Spiwoks-Becker et al. (31) reported results from synaptophysin knock-out mice suggesting that synaptophysin plays a role in synaptic vesicle recycling.

REFERENCES

1. Jahn, R., Schiebler, W., Ouimet, C., and Greengard, P. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4137–4141
2. Sudhof, T. C., Lottspeich, F., Greengard, P., Mehl, E., and Jahn, R. (1987) Science 238, 1142–1144
3. Knaus, P., Marqueze-Pouey, B., Scherer, H., and Betz, H. (1990) J. Cell Biol. 131, 1801–1809
4. McMahon, H. T., Bolshakov, V. Y., Janz, R., Hammer, R. E., Siegelbaum, S. A., and Sudhof, T. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4760–4764
5. Edelmann, L., Hanson, P. I., Chapman, E. R., and Jahn, R. (1995) J. Cell Biol. 131, 1801–1809
6. Bacci, A., Coco, S., Pravettoni, E., Schenk, U., Armano, S., Frassoni, C., Verderio, C., De Camilli, P., and Matteoli, M. (2001) J. Neurosci. 21, 6588–6596
7. Slepnev, V. I., and De Camilli, P. (2000) J. Neurosci. 21, 6588–6596
8. Liu, J. P., Sim, A. T., and Robinson, P. J. (1994) Science 265, 970–973
9. Cousin, M. A., and Robinson, P. J. (2000) J. Neurosci. 20, 949–957
10. Spiwoks-Becker, I., Vollrath, L., Seeliger, M. W., Jaisle, G., Eshkind, L. G., and Leube, R. E. (2001) Neuroscience 107, 127–142
11. Eshkind, L. G., and Leube, R. E. (1995) Cell Tissue Res. 282, 423–433
12. Edelmann, L., Hanson, P. I., Chapman, E. R., and Jahn, R. (1995) J. Cell Biol. 131, 1824–231
13. Bacci, A., Coco, S., Pravettoni, E., Schenk, U., Armano, S., Frassoni, C., Verderio, C., De Camilli, P., and Matteoli, M. (2001) J. Neurosci. 21, 6588–6596
14. Thiele, C., Hannah, M. J., Fahrenholz, F., and Huttner, W. B. (2000) Nat. Cell Biol. 2, 42–49
15. Hinshaw, J. E. (2000) Annu. Rev. Cell Dev. Biol. 16, 483–519
16. Dally, C., Sugimori, M., Moreira, J. E., Ziff, E. B., and Llinas, R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6120–6125
17. Sudhof, T. C., Lottspeich, F., Greengard, P., Mehl, E., and Jahn, R. (1987) Nucleic Acids Res. 15, 9677
18. Huttner, W. B., Schiebler, W., Greengard, P., and De Camilli, P. (1983) J. Cell Biol. 96, 1374–1388
19. Prekeris, R., and Terrian, D. M. (1997) J. Cell Biol. 137, 1589–1601
20. de Heuvel, E., Belt, A. W., Ramjaun, A. R., Wong, K., Sossin, W. S., and McPherson, P. S. (1997) J. Biol. Chem. 272, 8710–8716
21. Dally, C., and Ziff, E. B. (1997) J. Neurosci. 17, 2365–2375
22. Llinas, R., Sugimori, M., and Silver, R. B. (1992) Science 256, 677–679
23. Sheng, Z. H., Westenbroek, R. E., and Catterall, W. A. (1998) J. Bioenerg. Biomembr. 30, 335–345
24. Liu, J. P., Zhang, Q. X., Baldwin, G., and Robinson, P. J. (1996) J. Neurochem. 66, 2074–2084
25. Damke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) J. Cell Biol. 127, 915–934
26. Hinshaw, J. E., and Schmid, S. L. (1995) Nature 374, 190–192
27. Slepnev, V. I., and De Camilli, P. (2000) Nat. Rev. Neurosci. 1, 161–172
28. Robinson, P. J., Sontag, J. M., Liu, J. P., Frykse, E. M., Slaughter, C., McMahon, H., and Sudhof, T. C. (1993) Nature 365, 163–166
29. Liu, J. P., Sim, A. T., and Robinson, P. J. (1994) Science 265, 970–973
30. Cousin, M. A., and Robinson, P. J. (2000) J. Neurosci. 20, 949–957
31. Spiwoks-Becker, I., Vollrath, L., Seeliger, M. W., Jaisle, G., Eshkind, L. G., and Leube, R. E. (2001) Neuroscience 107, 127–142
Ca\textsuperscript{2+}-dependent Formation of a Dynamin-Synaptophysin Complex: POTENTIAL ROLE IN SYNAPTIC VESICLE ENDOCYTOSIS

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J. Biol. Chem. 2002, 277:9010-9015.
doi: 10.1074/jbc.M110815200 originally published online January 4, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M110815200

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