Stimulus-Dependent Dynamic Homo- and Heteromultimerization of Synaptobrevin/VAMP and Synaptophysin

Mikhail V. Khvotchev and Thomas C. Südhof*

Center for Basic Neuroscience, Department of Molecular Genetics and Howard Hughes Medical Institute, UT Southwestern Medical Center, Dallas, Texas 75390

Received August 9, 2004; Revised Manuscript Received September 20, 2004

ABSTRACT: Synaptophysin and synaptobrevin/VAMP are abundant synaptic vesicle proteins that form homo- and heterooligomers. We now use chemical cross-linking in synaptosomes, pinched-off nerve terminals that are capable of stimulus-dependent neurotransmitter release, to investigate whether these complexes are regulated. We show that in synaptosomes treated with three stimuli that induce exocytosis (a depolarizing K^+ solution, the excitatory neurotoxin α-latrotoxin, or the Ca^{2+}-ionophore ionomycin), the homo- and heteromultimerization of synaptophysin and synaptobrevin is increased up to 6-fold. Whereas at rest less than 10% of the total synaptobrevin and synaptophysin could be chemically cross-linked into homo- and heteromeric complexes, after stimulation up to 25% of synaptobrevin and synaptophysin are present in homo- and heteromultimers, suggesting that a large fraction of these synaptic vesicle proteins physiologically participate in such complexes. The increase in multimerization of synaptophysin and synaptobrevin was only observed in intact but not in lysed synaptosomes and could not be inhibited by general kinase or phosphatase inhibitors. The stimulus dependence of synaptophysin and synaptobrevin multimers indicates that the complexes are not composed of a fixed multisubunit structure, for example, as an ion channel, but represent distinct functional states of synaptobrevin and synaptophysin that are modulated in parallel with synaptic vesicle exo- and endocytosis.

In presynaptic nerve terminals, Ca^{2+} influx triggers neurotransmitter release by synaptic vesicle exocytosis (*1*). After exocytosis, synaptic vesicles undergo endocytosis and recycling. Synaptic vesicles are specialized secretory organelles with a unique protein composition (reviewed in ref *2*). One of the most abundant synaptic vesicle proteins is the soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptor (SNARE) protein synaptobrevin/VAMP. During exocytosis, synaptobrevin associates with the plasma membrane SNARE proteins syntaxin and synaptosomal-associated protein 25 (SNAP-25) to form a tight complex, the so-called core complex. This complex is generally thought to mediate, at least in part, synaptic vesicle fusion, although the precise role of the SNARE complex in membrane fusion remains unclear (reviewed in refs *3*−*5*). In addition to participating in the SNARE complex, synaptobrevin also associates into homodimers (*6*−*9*). Formation of synaptobrevin homodimers is mediated by a conserved amino acid motif of the transmembrane region, suggesting that it could drive oligomerization of the SNARE complex in vivo, contribute to SNARE complex stability, and enhance lipid mixing during fusion (*8*−*11*). Quantitation of the synaptobrevin dimer after detergent solubilization indicated a dissociation constant of ∼10 μM (*9*). Although this affinity appears to be a rather low for a physiologically significant interaction, synaptobrevin dimers are clearly present in isolated synaptic vesicles (*12*).

In addition to participating in SNARE complexes and homodimerization, synaptobrevin forms heterooligomers with synaptophysin, another abundant synaptic vesicle protein (*6*, *7*, *13*, *14*). Synaptobrevin associates with both of the two closely related synaptophysins that are present on synaptic vesicles, synaptophysin 1 and synaptophysin 2/synaptoporin (*15*−*17*). Synaptobrevin cannot simultaneously bind to synaptophysins and participate in the SNARE complex, suggesting that the binding of synaptobrevin to synaptophysin serves to restrict the availability of synaptobrevin for fusion (*7*, *17*). The synaptobrevin/synaptophysin complex is upregulated during development in vivo (in rats, ref *17*) and in vitro (in cultured neurons, ref *18*). Chronic blockade of glutamate receptors caused an increase in neurotransmitter release but a decrease in the synaptobrevin/synaptophysin complex (*19*). In contrast, the abundance of the complex was increased in a kindling model of epilepsy that induces neuronal hyperexcitability (*20*). Calakos and Scheller (*6*) detected the complex in neuroendocrine PC12 cells, whereas Becher et al., (*18*) found the complex to be absent from neuroendocrine cells. The protein sequences that mediate formation of the synaptobrevin/synaptophysin complex are not known. The fact that synaptobrevin cannot simultaneously bind to synaptophysin and form SNARE complexes (*6*) implicated cytoplasmic regions of these proteins and gave rise to the notion that the synaptophysin/synaptobrevin heterodimer regulates the availability of synaptobrevin for SNARE complex formation during fusion because it competes with SNARE complex formation (*7*). However, no direct binding of recombinant synaptophysin and synaptobrevin was reported.

* Corresponding author. Mailing address: Center for Basic Neuroscience, UT Southwestern, 6000 Harry Hines Blvd., Dallas, TX 75390-9111. Tel: (214) 648-1876. Fax: (214) 648-1879. E-mail: Thomas.Sudhof@UTSouthwestern.edu.

10.1021/bi048290+ CCC: $27.50
Published on Web 11/04/2004

Finally, in addition to forming complexes with synaptobrevin, synaptophysin associates into homooligomers via its transmembrane regions (*13*, *21*). Different from synaptobrevin, which also homomultimerizes via its transmembrane region but produces only homodimers, synaptophysin associates into hexamers and larger oligomers (*13*, *21*). These oligomers are noncovalent in situ but upon storage are converted into covalent oligomers that are linked by disulfide bonds. Physiologically, synaptophysin only has intramolecular disulfide bonds in its intravesicular loops, but these disulfide bonds are unstable and readily exchanged between neighboring molecules in the synaptophysin multimers (*13*). The synaptophysin multimer was proposed to form a stationary fixed protein complex that functions as a multimeric channel analogous to a gap junction (*21*).

Viewed together, the synaptic vesicle proteins synaptobrevin and synaptophysin thus engage in at least three complexes on the surface of synaptic vesicles: separate homooligomers of synaptophysin and synaptobrevin and heterooligomers between them. Do these oligomers represent stationary protein complexes, or are they dynamically regulated? If so, do they reflect different release states of nerve terminals or correlate with distinct stages of the vesicle cycle? One approach to distinguish between these hypotheses is to examine for each of the various complexes whether it is dynamic, that is, can be regulated by stimulation, or stationary, that is, invariant upon stimulation. Such experiments have been performed for the synaptobrevin/synaptophysin heterodimer and for the synaptophysin homomultimer (*22*, *23*). Using fluorescence resonance energy transfer (FRET)[1] in transfected neurons that express GFP-fusion proteins of synaptobrevin and synaptophysin, Pennuto et al. (*22*) showed that stimulation with the neurotoxin α-latrotoxin induced a decrease in all complexes involving synaptophysin and synaptobrevin in "large synaptic boutons", but had no effect on "small synaptic boutons". Reisinger et al. (*23*) used immunoprecipitations to assess the effect of stimulation by α-latrotoxin or the Ca^{2+}-ionophore A23187 on the synaptophysin−synaptobrevin complex and also found a stimulus-dependent decrease. Although these two studies arrive at the same conclusion, both studies suffer from methodological limitations. The use of transfected GFP-fusion proteins, the functionality of which is unclear, the heterogeneity of the synapses, and the small signal-to-noise ratio made it difficult to evaluate the results of the FRET study. In the immunoprecipitation experiments, proteins are first solubilized by detergents, which can affect the abundance and stability of the homo- and heterooligomers in situ.

In the present study, we have revisited this issue with an independent approach and employed protein cross-linking in synaptosomes to measure the abundance of synaptophysin and synaptobrevin homo- and heteromultimers in their native state. Using this method, we tested whether the association of endogenous synaptophysin and synaptobrevin into homo- and heterooligomers is dynamically regulated upon stimulation. Our data demonstrate that strong stimulation leads to an increase in all three complexes—the synaptophysin and the synaptobrevin homomultimers and the synaptophysin/synaptobrevin heterodimer. Our results indicate that the homo- and heterooligomers of synaptophysin and synaptobrevin are not stationary protein complexes but are dynamically regulated in parallel with the synaptic vesicle cycle.

[1] Abbreviations: FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; DSP, dithiobis-succinimidyl propionate; DTSSP, 3,3′-dithiobis-sulfosuccinimidyl propionate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

EXPERIMENTAL PROCEDURES

Preparation of Release-Competent Synaptosomes. Synaptosomes from mouse or rat forebrain were prepared essentially as described previously (*24*). All manipulations were performed at 0 °C. The tissue was dissected and homogenized in a glass−Teflon homogenizer at 900*g* in buffer A (0.32 M sucrose, 5 mM HEPES−NaOH, pH 7.4, 0.1 mM EDTA). Homogenates were cleared by low-speed centrifugation (1000*g* for 10 min) and centrifuged at 14 500*g* for 20 min to obtain the crude synaptosome fraction (P_2). The P_2 pellet was resuspended in 8.5% Percoll in buffer containing 0.25 M sucrose, 5 mM HEPES−NaOH, pH 7.4, and 0.1 mM EDTA and layered on the top of a 12%/20% Percoll step gradient in the same buffer. After centrifugation at 18 000*g* for 30 min, synaptosomes were recovered from the 12%/20% Percoll interface. Percoll was removed by addition of 30 vol of buffer A and centrifugation at 18 000*g* for 20 min. Purified synaptosomes (P_4) were resuspended in ice-cold gassed (95% O_2/5% CO_2) Krebs-bicarbonate buffer (composition in mM: 118 NaCl, 3.5 KCl, 1.25 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 5 HEPES−NaOH, pH 7.4, 11.5 glucose) typically at 4 mL per brain and used immediately.

Stimulation and Chemical Cross-Linking Experiments. Aliquots of synaptosomes were diluted 2-fold with Krebs-bicarbonate buffer (basal condition) or with stimulation buffer. Three stimulation conditions were employed: (a) high K^+ in modified Krebs-bicarbonate buffer containing 50 mM KCl and 71.5 mM NaCl; (b) ionomycin diluted from DMSO stock in Krebs-bicarbonate buffer; (c) α-latrotoxin purified from the black widow spider venom glands in Krebs-bicarbonate buffer. Synaptosomes were incubated at 34 °C for 10 min in the atmosphere of 95% O_2/5% CO_2 and subjected to chemical cross-linking for 10 min under the same incubation conditions. Two cross-linkers were used at 0.3 mM final concentration: DSP from freshly prepared DMSO stock and DTSSP dissolved in water. After the incubations, excess of the cross-linker was quenched by adding Tris-HCl, pH 7.4 (0.1 M final concentration); synaptosomes were pelleted by centrifugation (3000*g* for 10 min) and resuspended in SDS−PAGE sample buffer without reducing agents.

SDS−PAGE, Immunoblotting, and Protein Quantitations. Tris−glycine SDS−PAGE and immunoblotting were performed as described previously (*25*, *26*). Standard immunoblotting was performed with enhanced chemiluminescence (ECL, Amersham). Quantitative immunoblotting was performed using radiolabeled ^{125}I secondary antibodies (Amersham), and signals were quantitated on PhosphorImager (Molecular Dynamics) using ImageQuant software (*27*).

RESULTS

Cross-Linking Analysis of Synaptobrevin and Synaptophysin Homo- and Heterodimers. We purified synaptosomes on Percoll gradients (*28*) and incubated them at 34 °C in control buffer or buffers containing either high K^+ (to

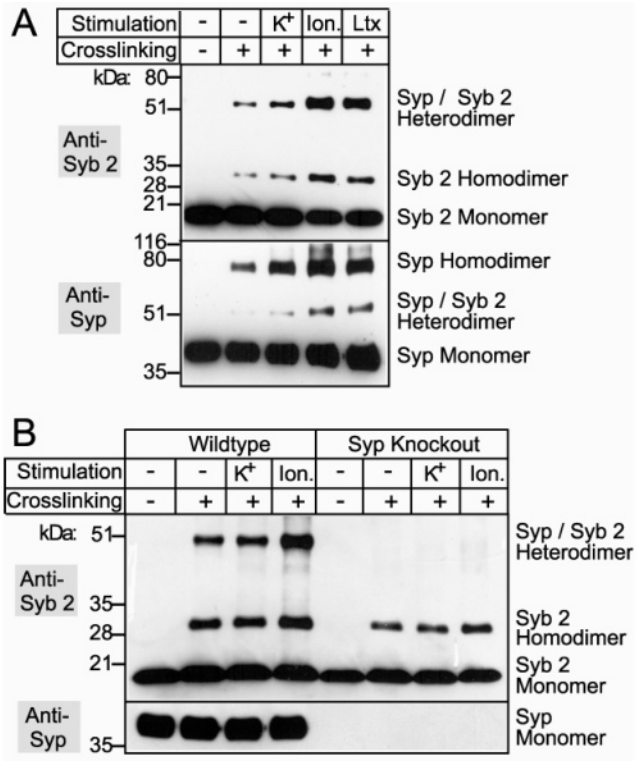

FIGURE 1: Immunoblotting analysis of synaptobrevin 2 (Syb 2) and synaptophysin (Syp) complexes after chemical cross-linking in synaptosomes. In panel A, synaptosomes were incubated for 10 min in oxygenated regular buffer or buffer containing a depolarizing concentration of K^+ (25 mM), 5 μM ionomycin (Ion.), or 4 nM α-latrotoxin (Ltx). Synaptosomes were treated with the membrane-permeable cross-linking agent DSP at 0.3 mM for 10 min, and synaptosomal proteins were analyzed by SDS−PAGE and immunoblotting with synaptobrevin (upper panel) and synaptophysin (lower panel) antibodies. Complexes are identified on the right. In panel B, synaptosomes prepared from wild-type and synaptophysin KO mice were analyzed as described for panel A.

stimulate the synaptosomes by depolarization-induced Ca^{2+} influx), ionomycin (to stimulate the synaptosomes by ionophore-mediated Ca^{2+} influx), or α-latrotoxin (a presynaptic excitatory toxin that directly stimulates synaptic vesicle exocytosis). Neurotransmitter release induced by these three agents was extensively characterized in previous studies (see ref *29* for an example of such experiments from our laboratory). After 10 min of incubation, we added 0.3 mM DSP (a membrane-permeable cross-linking agent) to the synaptosomes. This concentration of DSP was chosen because preliminary experiments showed that 0.3 mM DSP was the minimal effective concentration that induced reliable cross-linking of synaptobrevin and synaptophysin in intact synaptosomes (data not shown). After an additional incubation period of 10 min, we examined the cross-linking of synaptobrevin and synaptophysin in the synaptosomes by SDS−PAGE and immunoblotting.

In nonstimulated synaptosomes, chemical cross-linking uncovered the presence of synaptophysin homodimers, synaptobrevin homodimers, and synaptobrevin/synaptophysin heterodimers (Figure 1A). Quantitation using ^{125}I-labeled secondary antibodies revealed that approximately 2−5% and 1−2% of the total synaptobrevin 2 and synaptophysin, respectively, were engaged in homodimers under basal conditions. Similarly, approximately 2−5% and 0.5−1.0% of the total synaptobrevin 2 and synaptophysin, respectively, participated in synaptophysin/synaptobrevin heterodimers. Thus, in agreement with previous studies (*6*−*8*, *13*, *21*), a substantial proportion of synaptophysin and synaptobrevin were present in homo- and heteromeric complexes in nonstimulated synaptosomes. No cross-linking of synaptobrevin to other SNARE proteins was observed under our experimental conditions, presumably because of the poor efficiency of DSP in cross-linking SNARE complexes, which are best detected by immunoprecipitation experiments.

Stimulation dramatically increased the abundance of the synaptophysin and synaptobrevin homodimers and the synaptobrevin/synaptophysin heterodimers (Figure 1A). K^+ depolarization was the least effective, whereas both ionomycin and α-latrotoxin were very potent in increasing all three complexes. The increase in the synaptobrevin/synaptophysin heterodimer upon stimulation of exocytosis is somewhat surprising given the notion that this complex may represent a negative regulator of synaptobrevin for SNARE complex formation (*7*, *17*). This increase is also at odds with observations of transfected synaptobrevin- and synaptophysin-fusion proteins (*22*) and immunoprecipitation studies from cultured neurons (*23*), both of which suggested that the synaptobrevin/synaptophysin complex may decrease upon α-latrotoxin stimulation. To ensure that the cross-linked 50 kDa band observed with synaptophysin and synaptobrevin antibodies indeed corresponds to the synaptobrevin/synaptophysin heterodimer, we compared synaptosomes derived from wild-type and synaptophysin-deficient (knockout = KO) mice (*30*). In the synaptosomes from synaptophysin KO mice, no 50 kDa cross-linked band was detected with synaptobrevin antibodies, whereas the 25 kDa synaptobrevin homodimer was still present (Figure 1B), confirming that the cross-linking experiments monitor an increase in the synaptophysin/synaptobrevin heterodimer upon stimulation.

Quantitative Analysis of Homo- and Heteromultimerization. To measure the stimulus-dependent changes in synaptophysin and synaptobrevin multimerization, we quantified immunoblotting signals on gels using ^{125}I-labeled secondary antibodies (*27*). This approach allows precise calculation of the percentage of a given protein that is cross-linked because the relative abundance of the homo- and heterodimeric complexes and monomers are determined on the same blot. Quantitation of the homo- and heteromultimers of synaptobrevin 2 (Figure 2) showed that K^+ depolarization induced a modest increase in homodimerization of synaptobrevin (20% increase) and a larger increase in heterodimerization of synaptobrevin with synaptophysin (>100% increase). Stimulation with even low concentrations of ionomycin (0.5 μM) induced a much bigger increase in synaptobrevin homo- and heterodimerization, namely, a ∼100% increase in the synaptobrevin homodimer and a >500% increase in the synaptobrevin/synaptophysin heterodimer (Figure 2).

We next systematically examined the stimulus-dependent formation of all three synaptobrevin and synaptophysin complexes under the same conditions using antibodies to both synaptobrevin and synaptophysin (Figure 3). Ionomycin and α-latrotoxin similarly promoted multimerization of all complexes, but the three complexes were increased to different degrees. With a maximally 2-fold increase, the synaptobrevin homodimer was the least dynamic complex, whereas with an almost 10-fold increase, the synaptophysin homodimer was the most dynamic (Figure 3). Stimulation enhanced the

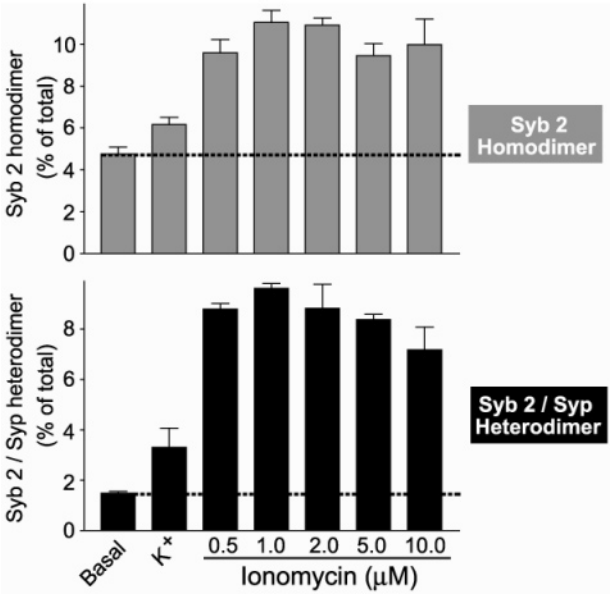

FIGURE 2: Quantitative immunoblotting analysis of stimulus-dependent synaptobrevin cross-linking in synaptosomes. Synaptosomes were treated with control buffer (basal), depolarizing K^+ buffer (25 mM), or various concentrations of ionomycin. The amount of free synaptobrevin and of synaptobrevin present in cross-linked homodimers and heterodimers with synaptophysin was measured by quantitative immunoblotting using ^{125}I-labeled secondary antibodies and phosphorimager detection. The amount of cross-linked synaptobrevin is expressed as percent of the total signal. Data are means of triplicates ± SEMs from a single experiment repeated three times with similar results.

synaptobrevin/synaptophysin heterodimer ~5-fold independent of whether it was measured with synaptobrevin or synaptophysin antibodies (Figure 3). The absolute percentage of cross-linked synaptobrevin and synaptophysin varied between synaptosome preparations, but the relative potentiation of multimerization by ionomycin or α-latrotoxin was highly reproducible. Within each experiment, approximately two times as much synaptobrevin reproducibly participated in synaptophysin heterodimers than in synaptobrevin homodimers, and the degree of stimulation of the latter exceeded that of the former.

Mechanisms of Homo- and Heteromultimerization. The differential dynamics of the synaptobrevin and synaptophysin homodimers and the synaptobrevin/synaptophysin heterodimer indicate that these complexes are fundamentally different. A very small part of the synaptobrevin sequence and a larger part of the synaptophysin sequences are intravesicular (*31*, *32*) and are exposed on the cell surface during exocytosis. When we applied a membrane-impermeable cross-linking agent, DTSSP, to synaptosomes, we observed a small but significant amount of synaptobrevin homodimers, whereas we detected no synaptobrevin/synaptophysin heterodimers (Figure 4A). Cross-linking of the homodimers was still enhanced by stimulation with ionomycin. Quantitation revealed that although the amount of cross-linking was decreased, the degree of enhanced cross-linking by ionomycin was similar (Figure 4B). This result supports the notion that the synaptobrevin homodimers are at least partially exposed at the cell surface during the vesicle lifecycle, whereas the synaptophysin/synaptobrevin heterodimers are not.

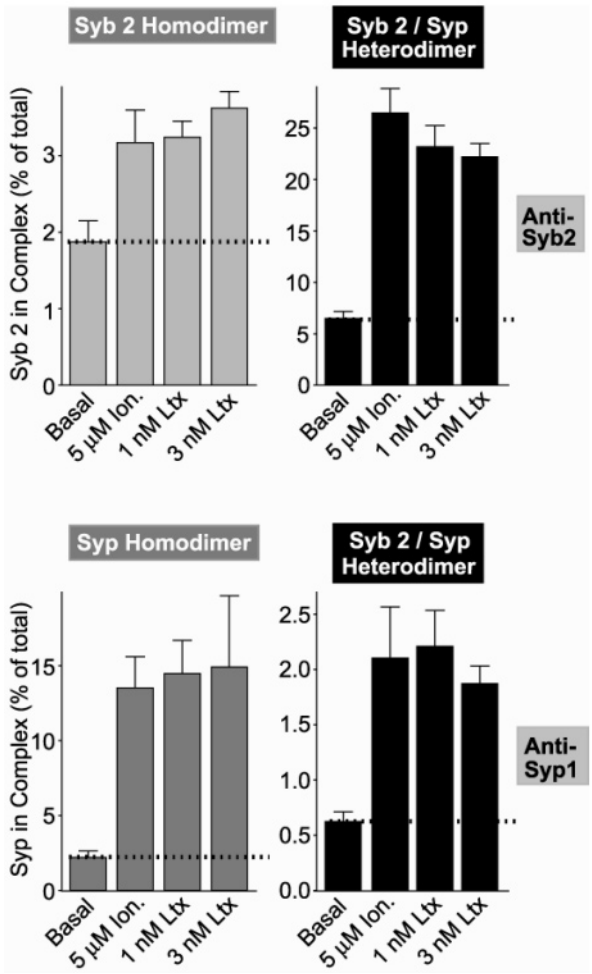

FIGURE 3: Comparison of stimulus-dependent synaptobrevin and synaptophysin complex formation. Synaptosomes were treated with control buffer (basal) or the indicated stimuli (Ion. = ionomycin; Ltx = α-latrotoxin) and subjected to cross-linking. Protein complexes were quantified using synaptobrevin antibodies (top) or synaptophysin antibodies (bottom) as described for Figure 2.

What mechanisms are involved in the stimulus-dependent modulation of the complexes? Hypotonic lysis of synaptosomes without detergent solubilization strongly inhibited the formation of synaptobrevin homodimers and synaptobrevin/synaptophysin heterodimers (Figure 5). Furthermore, in contrast to intact synaptosomes, in lysed synaptosomes ionomycin treatment did not induce an increase in the multimerization of synaptobrevin and synaptophysin. The apparent decrease in the synaptobrevin/synaptophysin heterodimer in lysed synaptosomes that were treated with ionomycin in the presence of calcium (Figure 5, last lane) could be due to increased proteolysis of synaptophysin, synaptobrevin, or both or could be caused by a reduced stability of the heterodimer after lysis of synaptosomes. Therefore, efficient multimerization of synaptobrevin and synaptophysin requires not only colocalization of both proteins in the synaptic vesicle membrane but also an intact cytosolic environment. This conclusion is further supported by previous studies demonstrating that the synaptophysin−synaptobrevin complex is upregulated during development and depends on an unidentified cytosolic factor present in the adult synaptosomes (*17*, *18*).

Protein complexes at synapses are often regulated by activity-dependent protein phosphorylation or dephospho-

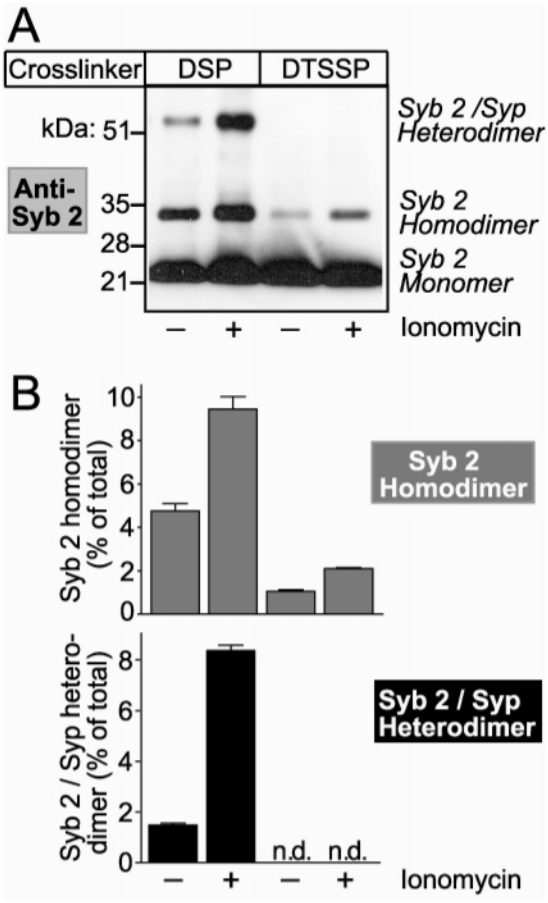

FIGURE 4: A membrane-impermeable cross-linking agent selectively detects synaptobrevin homodimers. Synaptosomes were incubated with control buffer or 5 μM ionomycin and treated with the membrane-permeable cross-linking agent DSP or the membrane-impermeable cross-linking agent DTSSP at 0.3 mM. Complexes were analyzed by SDS−PAGE (A) and quantitated by immunoblotting with synaptobrevin primary and ^{125}I-labeled secondary antibodies (B) as described for Figure 2.

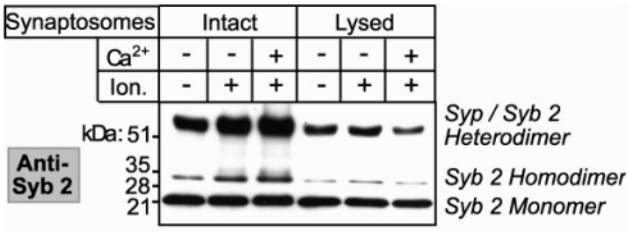

FIGURE 5: Synaptobrevin and -physin multimerization requires an intact cytosolic environment. Synaptosomes were treated with buffers containing the indicated components (Ca^{2+} = 1.25 mM Ca^{2+}; Ion. = 5 μM ionomycin) and pelleted. Synaptosomal pellets were resuspended in normal buffer or water (for hypotonic lysis) and incubated with DSP. Protein complexes were then examined by SDS−PAGE and immunoblotting with synaptobrevin antibodies.

rylation. To test whether serine/threonine kinases or phosphatases modulate synaptobrevin homo- and heterodimerization, we employed inhibitors of protein kinases or phosphatases. We examined whether synaptobrevin homo- and heterodimerization is altered by application of staurosporine, a general protein kinase inhibitor, or okadaic acid, a protein phosphatase inhibitor that at the high concentration used in our experiments acts on multiple phosphatases (Figure 6). However, neither agent caused a significant change in the abundance of the synaptobrevin/synaptophysin

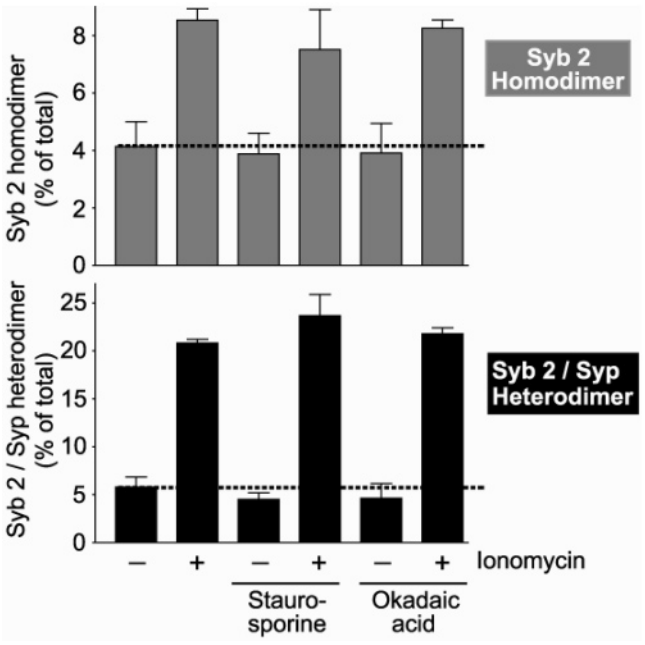

FIGURE 6: Synaptobrevin homo- and heteromultimerization are not inhibited by a protein kinase (staurosporine) or phosphatase inhibitor (okadaic acid). Synaptosomes were incubated for 10 min with or without 1 μM staurosporine or 5 μM okadaic acid, followed by additional incubation in the presence or absence of 5 μM ionomycin as indicated and cross-linking. Complexes were quantitated by SDS−PAGE and immunoblotting with synaptobrevin primary and ^{125}I-labeled secondary antibodies as described on Figure 2. Data are from a single experiment repeated two times.

complexes under resting or stimulation conditions, suggesting that serine/threonine kinases and phosphatases are not directly involved in synaptobrevin or synaptophysin multimerization. In addition, we tested effects of various pharmacological agents that inhibit specific protein serine/threonine kinases (ML-7, Gö 6976), tyrosine kinases and phosphatases (genistein, peroxovanadate), and lipid kinases (wortmanin, LY 294002), affect protein phosphorylation indirectly (forskolin, IBMX, phorbol esthers), or inhibit cytoskeleton assembly or disassembly (latrunculin A, jasplakinolide). Again, none of these agents had a significant effect on synaptobrevin/synaptophysin complexes (data not shown).

DISCUSSION

This study investigates three complexes formed by the synaptic vesicle proteins synaptobrevin and synaptophysin: the synaptobrevin homodimer, synaptophysin homomultimers, and the synaptobrevin/synaptophysin heterodimer. In a direct comparative analysis of these three complexes, our results confirm previous conclusions that these complexes exist physiologically, a question that was most recently debated for the synaptobrevin dimer (*9*, *12*). In fact, the abundance of all three complexes, as determined by the relatively inefficient chemical cross-linking approach in intact synaptosomes, is surprising. Our results suggest that the majority of synaptobrevin and synaptophysin are engaged in homo- and heteromeric complexes in vivo. A relatively larger percentage of synaptobrevin participated in heteromeric complexes with synaptophysin than in homomeric complexes; conversely, a greater percentage of synaptophysin participated in homomultimers than in heterodimers with synaptobrevin. The most important result of our study,

however, may be the finding that stimulation regulates the formation of all three complexes. Although the relative abundance of the three complexes and their changes upon stimulation differed, all complexes were significantly upregulated when synaptosomes were stimulated. In fact, in some experiments stimulation resulted in the cross-linking of up to a third of the total synaptobrevin into heteromultimeric complexes with synaptophysin and of up to a fourth of the total synaptophysin into homomeric complexes. The dynamic nature of the complexes implies that they are not stationary components of vesicles but are regulated in parallel with synaptic vesicle exo- and endocytosis.

A limitation of our experimental approach is that we used synaptosomes, an artificial system that may not faithfully reflect all properties of normal nerve terminals in an intact brain. A second limitation is that we employed chemical cross-linking as a method to identify complexes. This technique can only measure a fraction of protein complexes that exist in situ and usually underestimates the abundance of such complexes. Indeed, we observed no cross-linking of synaptobrevin into SNARE complexes, probably because these complexes are resistant to chemical cross-linking by DSP or DTSSP. As a result, we do not know whether homomultimerization of synaptobrevin via its transmembrane region is related to SNARE complex formation. However, the limitations inherent in using synaptosomes and chemical cross-linking are balanced by major advantages of this approach. As release-competent subcellular structures that are freshly prepared from brain, the regulation of synaptic vesicles is not necessarily less physiological in synaptosomes than, for example, in cultured neurons in which the nerve terminals have developed under relatively nonphysiological conditions. Similarly, the use of chemical cross-linking allows monitoring of complexes formed by endogenous, nonmodified proteins and does not require disrupting functional nerve terminals prior to analysis. Thus, although our measurements of the various synaptobrevin and synaptophysin complexes only allow lower limit estimates of their abundance, these measurement likely report on a physiological event.

Two previous studies examined the effect of stimulation on synaptobrevin and synaptophysin complexes. Pennuto et al. (*22*) used FRET between transfected EYFP- and ECFP-fusion proteins to monitor these complexes. With this elegant method, Pennuto et al. found that α-latrotoxin stimulation decreased the abundance of the synaptobrevin/synaptophysin heterodimer. Reisinger et al. (*23*) employed immunoprecipitations of proteins solubilized from cultured neurons (treated with control buffer or α-latrotoxin) as a method to monitor the synaptobrevin/synaptophysin complex and also observed a stimulation-dependent decrease in the complex. At present, it is difficult to resolve the discrepancy between our study, which reports a stimulation-dependent increase in this complex, and the previous results, which suggest a stimulation-dependent decrease. However, the two previous studies suffered from limitations that restrict their usefulness. In the FRET study (*22*), an effect of stimulation on the synaptobrevin and synaptophysin complexes was only observed in a subset of synapses that were particularly large, and the FRET signals were rather small, presumably because the fluorophores on the fusion proteins are not in close proximity. Moreover, it is unclear whether the EYFP- and ECFP-fusion proteins of synaptobrevin and synaptophysin used in this study were functionally integrated into the secretory machinery of the nerve terminal. This is particularly a concern for synaptophysin, the overexpression of which in PC12 cells blocks exocytosis (*33*), suggesting the possibility that transfected synaptophysin−ECFP alters exocytosis in the nerve terminals. Preliminary data from our lab support this possibility (M. V. K. and T. C. S., unpublished). In the immunoprecipitation study, proteins first had to be solubilized by detergents before an analysis could be performed. Since simple lysis of synaptosomes already greatly diminishes the abundance of both synaptobrevin complexes (Figure 5), it is unclear whether the immunoprecipitated complexes retain the physiological assembly states of the protein complexes in situ. In fact, our data demonstrating that the complexes depend on an intact cellular environment indicate that one reason for the stimulation-dependent decrease observed in previous studies may have been that the strong stimulations used impaired the integrity of the cells.

What is the functional role of the complexes studied here? The synaptobrevin/synaptophysin complex appears to prevent synaptobrevin from entering into SNARE complexes (*6*, *7*), suggesting that the complex regulates the availability of synaptobrevin for fusion. However, deletion of synaptophysin in mice does not appear to have a major effect on neurotransmitter release (*30*, *34*), arguing against a role for synaptophysin in SNARE complex assembly. Unfortunately at this point no clear function for synaptophysin has emerged despite intense studies. It seems likely that synaptophysin as one of the most abundant synaptic vesicle proteins—possibly the most abundant vesicle protein—performs a major role in vesicle traffic, which could have easily been missed in the electrophysiological analysis of brain slices from the synaptophysin knockout mice (*30*, *35*). Until this function is defined, an evaluation of the role of the synaptophysin homo- and heteromeric complexes will be difficult, although their striking stimulus-dependent modulation shown here strongly argues for a role in synaptic vesicle membrane traffic.

The formulation of a functional hypothesis for a possible role of the synaptobrevin homodimer is much easier than for the synaptophysin complexes. The action of the SNARE protein synaptobrevin in synaptic vesicle fusion is thought to involve the transmembrane region of synaptobrevin (reviewed in ref *5*). Homodimerization of synaptobrevin via its transmembrane region (*8*−*10*, *12*) likely promotes fusion by facilitating multimerization of SNARE complexes and assembly of fusion pores. The stimulus-dependent increase in the synaptobrevin homodimer could thus reflect an upregulation of the "fusability" of synaptic vesicles or an assembly of such homodimers during fusion, a possibility that is supported by the detection of such homodimers with nonpermeable cross-linking agents (Figure 4). Differentiating between these two interesting possibilities will require a mutational analysis of synaptobrevin in a rescue system that allows testing of structure−function relations of synaptobrevin in vivo.

ACKNOWLEDGMENT

We would like to thank I. Kornblum and E. Borowicz for excellent technical assistance.

REFERENCES

1. Katz, B. (1969) The release of neural transmitter substances, Liverpool University Press, Liverpool, U.K.
2. Südhof, T. C. (1995) The synaptic vesicle cycle: a cascade of protein−protein interaction, *Nature 375*, 488−493.
3. Söllner, T. H., and Rothman, J. E. (1996) Molecular machinery mediating vesicle budding, docking and fusion, *Experientia 52*, 1021−1025.
4. Chen, Y. A., and Scheller, R. H. (2001) SNARE-mediated membrane fusion, *Nat. Rev. Mol. Cell Biol. 2*, 98−106.
5. Jahn, R., Lang, T., and Südhof, T. C. (2003) Membrane fusion, *Cell 112*, 519−533.
6. Calakos, N., and Scheller, R. (1994) Vesicle-associated membrane protein and synaptophysin are associated on the synaptic vesicle, *J. Biol. Chem. 269*, 24534−24537.
7. Edelmann, L., Hanson, P. I., Chapman, E. R., and Jahn, R. (1995) Synaptobrevin binding to synaptophysin: a potential mechanism for controlling the exocytotic fusion machine, *EMBO J. 14*, 224−231.
8. Laage, R., and Langosch, D. (1997) Dimerization of the synaptic vesicle protein synaptobrevin (vesicle-associated membrane protein) II depends on specific residues within the transmembrane domain, *Eur. J. Biochem. 249*, 540−546.
9. Bowen, M. E., Engelman, D. M., and Brünger, A. T. (2002) Mutational analysis of synaptobrevin transmembrane domain oligomerization, *Biochemistry 41*, 15861−15866.
10. Laage, R., Rohde, J., Brosig, B., and Langosch, D. A. (2000) Conserved membrane-spanning amino acid motif drives homomeric and supports heteromeric assembly of presynaptic SNARE proteins, *J. Biol Chem. 275*, 17481−17487.
11. Fleming, K. G., and Engelman, D. M. (2001) Computation and mutagenesis suggest a right-handed structure for the synaptobrevin transmembrane dimer, *Proteins 45*, 313−317.
12. Roy, R., Laage, R., and Langosch, D. (2004) Synaptobrevin transmembrane domain dimerization − revisited, *Biochemistry 43*, 4964−4970.
13. Johnston, P. A., and Südhof, T. C. (1990) The multisubunit structure of synaptophysin. Relationship between disulfide bonding and homooligomerization, *J. Biol. Chem. 265*, 8869−8873.
14. Washbourne, P., Schiavo, G., and Montecucco, C. (1995) Vesicle-associated membrane protein-2 (synaptobrevin-2) forms a complex with synaptophysin, *Biochem. J. 305*, 721−724.
15. Knaus, P., Marquèze-Pouey, B., Scherer, H., and Betz, H. (1990) Synaptoporin, a novel putative channel protein of synaptic vesicles, *Neuron 5*, 453−462.
16. Fykse, E. M., Takei, K., Walch-Solimena, C., Geppert, M., Jahn, R., De Camilli, P., and Südhof, T. C. (1993) Relative properties and localizations of synaptic vesicle protein isoforms: The case of the synaptophysins, *J. Neurosci. 13*, 4997−5007.
17. Becher, A., Drenckhahn, A., Pahner, I., Margittai, M., Jahn, R., and Ahnert-Hilger, G. (1999) The synaptophysin-synaptobrevin complex: a hallmark of synaptic vesicle maturation, *J. Neurosci. 19*, 1922−1931.
18. Becher, A., Drenckhahn, A., Pahner, I., and Ahnert-Hilger, G. (1999) The synaptophysin-synaptobrevin complex is developmentally upregulated in cultivated neurons but is absent in neuroendocrine cells, *Eur. J. Cell Biol. 78*, 650−656.
19. Bacci, A., Coco, S., Pravettoni, E., Schenk, U., Armano, S., Frassoni, C., Verderio, C., De Camilli, P., and Matteoli, M. (2001) Chronic blockade of glutamate receptors enhances presynaptic release and downregulates the interaction between synaptophysin-synaptobrevin-vesicle-associated membrane protein 2, *J. Neurosci. 21*, 6588−6596.
20. Hinz, B., Becher, A., Mitter, D., Schulze, K., Heinemann, U., Draguhn, A., and Ahnert-Hilger, G. (2001) Activity-dependent changes of the presynaptic synaptophysin-synaptobrevin complex in adult rat brain, *Eur. J. Cell Biol. 80*, 615−619.
21. Thomas, L., Hartung, K., Langosch, D., Rehm, H., Bamberg, E., Franke, W. W., and Betz, H. (1988) Identification of synaptophysin as a hexameric channel protein of the synaptic vesicle membrane, *Science 242*, 1050−1053.
22. Pennuto, M., Dunlap, D., Contestabile, A., Benfenati, F., and Valtorta, F. (2002) Fluorescence resonance energy transfer detection of synaptophysin I and vesicle-associated membrane protein 2 interactions during exocytosis from single live synapses, *Mol. Biol. Cell 13*, 2706−2717.
23. Reisinger, C., Yelamanchili, S. V., Hinz, B., Mitter, D., Becher, A., Bigalke, H., and Ahnert-Hilger, G. (2004) The synaptophysin/synaptobrevin complex dissociates independently of neuroexocytosis, *J. Neurochem. 90*, 1−8.
24. Khvotchev, M., and Südhof, T. C. (1998) Newly synthesized phosphatidylinositol phosphates are required for synaptic norepinephrine but not glutamate or gamma-aminobutyric acid (GABA) release, *J. Biol. Chem. 273*, 21451−21454.
25. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
26. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350−4354.
27. Rosahl, T. W., Spillane, D., Missler, M., Herz, J., Selig, D. K., Wolff, J. R., Hammer, R. E., Malenka, R. C., and Südhof, T. C. (1995) Essential functions of synapsins I and II in synaptic vesicle regulation, *Nature 375*, 488−493.
28. Nagy, A., and Delgado-Escueta, A. V. (1984) Rapid preparation of synaptosomes from mammalian brain using nontoxic isoosmotic gradient material (Percoll), *J. Neurochem. 43*, 1114−1123.
29. Khvotchev, M., and Südhof, T. C. (2000) Alpha-latrotoxin triggers transmitter release via direct insertion into the presynaptic plasma membrane, *EMBO J. 19*, 3250−3262.
30. McMahon, H. T., Bolshakov, V. Y., Janz, R., Hammer, R. E., Siegelbaum, S. A., and Südhof, T. C. (1996) Synaptophysin, a major synaptic vesicle protein, is not essential for neurotransmitter release, *Proc. Natl. Acad. Sci. U.S.A. 93*, 4760−4764.
31. Trimble, W. S., and Scheller, R. H. (1988) Molecular biology of synaptic vesicle-associated proteins, *Trends Neurosci. 11*, 241−242.
32. Johnston, P. A., Jahn, R. and Südhof, T. C. (1989) Transmembrane topography and evolutionary conservation of synaptophysin, *J. Biol. Chem. 264*, 1268−1273.
33. Sugita, S., Janz, R., and Südhof, T. C. (1999) Synaptogyrins regulate Ca^{2+}-dependent exocytosis in PC12 cells, *J. Biol. Chem. 274*, 18893−18901.
34. Eshkind, L. G., and Leube, R. E. (1995) Mice lacking synaptophysin reproduce and form typical synaptic vesicles, *Cell Tissue Res. 282*, 423−433.
35. Janz, R., Südhof, T. C., Hammer, R. E., Unni, V., Siegelbaum, S. A., and Bolshakov, V. Y. (1999) Essential roles in synaptic plasticity for synaptogyrin I and synaptophysin I, *Neuron 24*, 687−700.

BI048290+