Evidence for structural and functional diversity among SDS-resistant SNARE complexes in neuroendocrine cells

Helmut Kubista*, **Hannah Edelbauer and Stefan Boehm**

Department of Pharmacology, University of Vienna, Waehringerstrasse 13a, 1090 Vienna, Austria

*Author for correspondence (e-mail: helmut.kubista@univie.ac.at)

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Summary

The core complex, formed by the SNARE proteins synaptobrevin 2, syntaxin 1 and SNAP-25, is an important component of the synaptic fusion machinery and shows remarkable in vitro stability, as exemplified by its SDS-resistance. In western blots, antibodies against one of these SNARE proteins reveal the existence of not only an SDS-resistant ternary complex but also as many as five bands between 60 and >200 kDa. Structural conformation as well as possible functions of these various complexes remained elusive.

In western blots of protein extracts from PC12 cell membranes, an antibody against SNAP-25 detected two heat-sensitive SDS-resistant bands with apparent molecular weights of 100 and 230 kDa. A syntaxin antibody recognized only the 230 kDa band and required heat-treatment of the blotting membrane to detect the 100 kDa band. Various antibodies against synaptobrevin failed to detect SNARE complexes in conventional western blots and detected either the 100 kDa band or the 230 kDa band on heat-treated blotting membranes.

When PC12 cells were exposed to various extracellular K^+-concentrations (to evoke depolarization-induced Ca^{2+} influx) or permeabilized in the presence of basal or elevated free Ca^{2+}, levels of these SNARE complexes were altered differentially: moderate Ca^{2+} rises (≤1 μM) caused an increase, whereas Ca^{2+} elevations of more than 1 μM led to a decrease in the 230 kDa band. Under both conditions the 100 kDa band was either increased or remained unchanged.

Our data show that various SDS-resistant complexes occur in living cells and indicate that they represent SNARE complexes with different structures and diverging functions. The distinct behavior of these complexes under release-promoting conditions indicates that these SNARE structures have different roles in exocytosis.

Key words: SNARE, PC12, Transmitter release, SNAP-25, Syntaxin, Synaptobrevin

Introduction

Members of the soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs) superfamily are abundantly and widely expressed proteins that are involved in all intracellular membrane fusion events. In neurons, the synaptic SNARE proteins syntaxin 1, SNAP-25, and synaptobrevin 2 contribute to Ca^{2+}-dependent vesicle exocytosis, as shown by the fact that cleavage of these proteins by clostridial neurotoxins prevents neurotransmitter release. Studies using these neurotoxins as well as genetic ablation studies demonstrated that SNARE proteins are not required for fusion per se (Schoch et al., 2001; Washbourne et al., 2002) but rather serve to provide a high Ca^{2+} sensitivity of the fusion process (Gerona et al., 2000; Stewart et al., 2000; Reim et al., 2001). For membrane fusion to occur, SNAREs must localize to opposing membranes and assemble into a protein complex to bring the two membranes into close apposition, thereby facilitating bilayer mixing. Thus, the ability of syntaxin 1, SNAP-25 and synaptobrevin 2 to form heteromeric complexes is believed to be the basis for neurotransmitter release (Jahn and Sudhof, 1999; Chen and Scheller, 2001).

The structure of the heterotrimeric SNARE complex was resolved in 1998 by Sutton and co-workers (Sutton et al., 1998). The cytosolic part of the complex consists of a parallel four-helices bundle (one helix each provided by syntaxin and synaptobrevin and two helices from the SNAP-25 molecule) with the transmembrane domains of synaptobrevin and syntaxin on the membrane proximal side and their N-termini on the membrane-distal side. The four helices form a coiled coil structure that provides the protein complex with a high thermal and chemical stability, for example, for sodium dodecyl sulfate (SDS)-resistance at temperatures up to ~80°C (Chen et al., 1999). In vivo, SNARE complexes can be disassembled only by the ATPase NSF and it is thought that this occurs in a post fusion step (Weber et al., 2000; Littleton et al., 2001). In the next round of the vesicle cycle, SNARE proteins would then be provided as monomers to form new SNARE complexes in the course of vesicle priming (reviewed in Rizo and Sudhof, 2002).

Because ternary SNARE complexes are so stable, they can be detected in western blots as bands that show immunoreactivity for antibodies directed against each of the

three SNARE proteins and which are lost when protein samples are boiled before the separation in SDS-containing polyacrylamide gels (Hayashi et al., 1994).

Interestingly, SNARE complexes do not migrate to only one position in SDS-PAGE, but were found at several different positions equivalent to molecular weights between ~60 and >200 kDa. Frequently, as many as three (Otto et al., 1997) or even five (Matveeva et al., 2003) bands were recognized by antibodies against synaptobrevin, SNAP-25 and syntaxin. The structural basis for multiple complexes is unknown. Some of these complexes were proposed to represent folding intermediates (Lawrence and Dolly, 2002). Slowly migrating complexes might be attributable to oligomeric forms of a ternary complex (Tokumaru et al., 2001). In addition to the structural issues, the functional meaning of biochemically identified SDS-resistant SNARE complexes also remained elusive. In permeabilized phaeochromocytoma 12 (PC12) cells, for example, the formation of SDS-resistant complexes is insufficient for transmitter release (Chen et al., 1999) whereas, in chromaffin cells, both vesicle release that did require SDS-resistant SNARE complexes, and that which did not, were identified (Xu et al., 1999). However, only one (presumably the most abundant) species of SDS-resistant SNARE complexes was investigated in these studies.

In living PC12 cells, a direct interaction between SNAP-25 and synaptobrevin was observed using fluorescence resonance energy transfer (Xia et al., 2001). Studies using botulinum neurotoxins B, E and C1 demonstrate that syntaxin, synaptobrevin and SNAP-25 are required for regulated exocytosis in this cell line (Gerona et al., 2000). In western blots of protein extracts of permeabilized PC12 cells, three SDS-resistant SNARE complexes (Banerjee et al., 1996a) were detected. Nevertheless, it remained unclear whether SDS-resistant SNARE complexes do exist in PC12 cells in vivo and whether they might change during vesicle exocytosis. To address this question, we investigated the levels of SDS-resistant SNARE complexes in PC12 protein extracts that were obtained under various release-promoting conditions. Our results, which have been presented in preliminary form (Kubista and Boehm, 2001; Kubista et al., 2002), indicate that PC12 cells contain only two SDS-resistant SNARE complexes, which differ in conformation or composition, and which can also be functionally distinguished.

Materials and Methods

Materials

PC12 cells were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Culture dishes were from Iwaki (Tokyo, Japan) and Nunc (Roskilde, Denmark). Rat tail collagen was from Biomedical Technologies (Stoughton, MA). OPTI-MEM I, horse serum, penicillin-streptomycin and L-glutamine were from Life Technologies (Vienna, Austria), fetal calf serum was from PAA Laboratories (Linz, Austria). PBS-EDTA was from Bi°Concept (Allschwil, Switzerland), ionomycin, fura-2/AM, Pluronic F-127 and protease inhibitor cocktail set III were from Calbiochem (San Diego, CA). Digitonin, Triton X-100, DTT and 2-mercaptoethanol were from Sigma (Vienna, Austria). Prestained molecular weight standards were from Sigma and BIO-RAD (Vienna, Austria). Horseradish peroxidase-linked antibody against mouse Ig was from Amersham (Piscataway, NJ), SuperSignal chemiluminescent substrate and the Micro BCA Protein Assay Reagent Kit were from Pierce (Rockford, IL). Electrophoresis apparatus and Semi-Dry blotter were from Bio-Rad (Vienna, Austria); nitrocellulose membrane was from Schleicher and Schuell (Dassel, Germany). (–)-[Ring-2,5,6-^{3}H]noradrenaline was obtained from NEN (Dreieich, Germany). Monoclonal antibodies directed against SNAP-25 (MAB331), syntaxin (MAB336) and synaptobrevin/VAMP (MAB333 and AB5856) were from Chemicon (Temecula, CA), monoclonal syntaxin antibody (clone HPC-1) was from Sigma (Vienna, Austria), monoclonal syntaxin1 antibody (clone 78.3) and monoclonal synaptobrevin2/VAMP2 antibody (clone 69.1) were from Synaptic Systems (Göttingen, Germany). Bulk chemicals were from Sigma (Vienna, Austria) and BIOMOL (Plymouth Meeting, PA).

Cell Culture

PC12 cells were plated onto collagen-coated culture dishes and were kept in OPTI-MEM I supplemented with 50.000 IU l^{-1} penicillin, 50 mg l^{-1} streptomycin, 2 mM L-glutamine, 10% horse serum and 5% fetal calf serum (OPTI-MEM-complete) at 37°C in 5% CO_2.

Cell cultures were split once a week and the medium was exchanged twice a week. Cultures of equal density (two dishes per experimental condition) were generated by seeding equal volumes (~5 to 7.5×10^6 cells) of a cell suspension into collagen-coated dishes of 90 mm in diameter. The cells were grown for 4 to 5 days in OPTI-MEM-complete, which was exchanged 48 hours before the experiments.

Incubation conditions and immunoblots

PC12 cells were washed once in physiological buffer [150 mM NaCl, 4 mM KCl, 1.5 or 10 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES (pH 7.4)] and then incubated at room temperature in the same buffer or in buffer with elevated K^+ concentrations (K^+ replaced an equimolar amount of Na^+) or in buffer designed to mimic the intracellular milieu [100 mM potassium gluconate, 10 mM EGTA, 1.24 mM $MgCl_2$, 1 mM DTT, 10 mM HEPES (pH 7.3) and $CaCl_2$ to obtain various concentrations of free Ca^{2+}] containing 10 μM digitonin to permeabilize the cells. Each experimental condition was applied to two dishes. At the end of the incubation, cells were lysed by replacing the extracellular buffer with 2 ml per dish of hypotonic buffer (10 mM $MgCl_2$, 10 mM Tris (pH 7.4), 2.5 mM EGTA and 1:500 protease inhibitor cocktail set III) and by freezing them immediately by adding 10 ml of liquid nitrogen to the hypotonic buffer. Cells were then thawed, scraped off the dishes and pooled with cells of the same treatment. Cell solutions were collected in 15 ml plastic tubes, frozen in liquid nitrogen, thawed and subjected to sonication. Cell membranes were collected from the lysed cell suspension by centrifugation at 500 ***g*** (5 minutes, 4°C) and subsequent centrifugation of the supernatant at 50,000 ***g*** (50 minutes, 4°C). Protein extraction was performed by resuspending the final membrane pellet in SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.01% Bromophenol Blue. In several experiments Bromophenol Blue was initially omitted to allow absorbance measurements of the membrane extracts at 280 nm but added later. This was done in order to verify that the amount of protein extracted did not vary substantially between individual samples. Samples were then incubated at different temperatures (at 37°C to detect SDS-resistant SNARE complexes, at 100°C to disrupt SDS-resistant SNARE complexes and at 40°C to 90°C to investigate the temperature dependence of these complexes) for 5 minutes and subjected to SDS-PAGE.

Although SNARE proteins in solution may form SDS-resistant SNARE complexes this does not occur in the presence of SDS (Hayashi et al., 1994; Otto et al., 1997). Therefore, membrane proteins as were extracted as quick as possible in SDS sample buffer after completion of the experiments. Moreover, SDS samples were immediately separated by SDS-PAGE and transferred to nitrocellulose membrane.

Triton X-100 extraction of membrane proteins was performed by resuspending the membranes 50 mM NaCl, 10 mM Tris-HCl (pH 7,4) and 1% Triton X-100. Insoluble material was removed by centrifugation at 50,000 ***g*** (50 minutes, 4°C). Protein concentration of Triton X-100 extracts was determined by using the Micro BCA Protein Assay Reagent Kit.

Direct lysis of PC12 cells in SDS was performed by resuspending a cell pellet in SDS sample buffer. The viscous solution was then passaged five times through a 27 G needle followed by sonication to obtain the fluidity required for loading the sample onto the gel.

SDS-PAGE) was performed on 5% (stacking) and 8% (separating) gels. After electrophoretic transfer of proteins to nitrocellulose membranes the immunoreactive material was analysed with antibodies against SNARE and horseradish peroxidase-linked secondary antibody using enhanced chemiluminescence. Luminescence signals were captured on X-ray film. Films were scanned and bands were analysed densitometrically using the Molecular Analyst software (Bio-Rad).

Heat-treatment of nitrocellulose membranes before western blotting was performed by exposing the membranes to hot (90°C) electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) for 3 minutes followed by incubation in the same buffer at room temperature for another 5 minutes.

Measurement of [^{3}H]noradrenaline release

Release of [^{3}H]noradrenaline was measured from PC12 cells cultured in 6-well plates to 75% confluency. Cultures were preincubated in OPTI-MEM-complete plus 0.01 μmol l^{-1} [^{3}H]noradrenaline (specific activity 56.4 Ci $mmol^{-1}$) and 1 mM ascorbic acid for 1 hour at 37°C in 5% CO_2. The cells were then washed once in physiological buffer (see above) and incubated for another hour in OPTI-MEM-complete at 37°C in 5% CO_2. Thereafter, cells were washed four times with physiological buffer and the last wash volume was retained to determine basal [^{3}H]noradrenaline release. Secretion was then stimulated by exposing the cells to various test solutions (as indicated). Test solutions were removed after the chosen incubation period and transferred to scintillation vials. Radioactivity that has remained in the cells was extracted by exposing the cultures to 2% (v/v) perchloric acid. Radioactivity of these extracts and of the collected test solutions was determined by liquid scintillation counting.

To find out the time course of [^{3}H]noradrenaline release, PC12 cells were plated onto 5 mm discs coated with rat tail collagen (Biomedical Technologies, Stoughton, MA), as previously described for sympathetic neurons (Scholze et al., 2002). After labelling with [^{3}H]noradrenaline, culture discs were transferred to small chambers and superfused with physiological buffer. Superfusion was performed at 25°C at a rate of ~1.0 ml min^{-1}. After a wash-out period (60 minutes) to remove excess radioactivity, superfusate fractions were collected for 4 minutes. The radioactivity retrieved within a 4 minutes fraction was calculated as percentage of the total radioactivity in the cultures.

Fura-2 Ca^{2+} fluorometry

PC12 cells grown in 35 mm collagen-coated dishes were loaded with indicator dye (fura2/AM) for 45 minutes at 37°C in 5% CO_2 in growth medium containing 5% horse serum and 5 μM fura-2/AM with 0.01% Pluronic F-127. Thereafter, cells were washed three times with physiological buffer. The dishes were mounted on an Axiovert 200 inverted microscope (Zeiss, Göttingen, Germany) equipped with a MultiSpec Micro-Imager (Optical Insights LLC, Santa Fe, NM) and a Polychrome IV monochromator (Till-Photonics, Gräfelfing, Germany). Components were controlled using the MetaFluor software (Universal Imaging, Downington, PA). Cells were superfused continuously via the DAD-12 drug application device (Adams and List, Westbury, NY). Fluorescence was measured at 510 nm with excitation at 340 and 380 nm. Minimum and maximum fura-2 fluorescence was determined in ionomycin-treated cells to calculate the free Ca^{2+} concentration from the fluorescence ratio according to the method published by Grynkiewicz et al., assuming a K_D of 224 nM for Ca^{2+} (Grynkiewicz et al., 1985).

Results

Transmitter release from PC12 cells requires syntaxin 1, synaptobrevin 2 and SNAP-25 (Gerona et al., 2000; Lang et al., 2002; Proux-Gillardeaux et al., 2003; Quetglas et al., 2002). To identify SDS-resistant complexes of these proteins in the PC12 cell line, membrane proteins were extracted in SDS-sample buffer without boiling, separated by SDS-PAGE, blotted and analysed by immunostaining with antibodies directed against syntaxin, synaptobrevin and SNAP-25. A range of commercially available antibodies always detected the appropriate monomer but detected none, one or two high molecular weight bands (Fig. 1A-C). The most intense signals for complexes were obtained with a monoclonal SNAP-25 antibody (MAB331). With this antibody, immunoreactive bands were visible in the ranges of 20-30, 80-110 and 190-240 kDa. As determined from the co-migration of molecular weight markers using intra- and extrapolation, the mean values of apparent molecular masses of these bands were 25, 100 and 230 kDa, respectively. These high molecular weight bands were not detected in samples that had been boiled prior to electrophoretic separation (Fig. 1A). Boiling of the samples led to an increased intensity of the band corresponding to monomeric SNAREs and this was also seen with antibodies that failed to detect any slowly migrating bands. Therefore, the SNARE proteins might be incorporated in the protein complexes in a manner that makes the epitope inaccessible for antibody binding. In line with this hypothesis, antibody MAB336 against syntaxin (αSTX^1) did not detect any slowly migrating bands, but boiling of the samples increased the band corresponding to monomeric syntaxin (Fig. 1A). However, slowly migrating bands were identified with another syntaxin antibody, clone HPC-1 (αSTX^2) that has its epitope in an extracellular domain of the antigen (Fig. 1B). Since the SDS-resistance of SNARE complexes is heat-sensitive (Hayashi et al., 1994), we tested whether (partial-) disassembly of complexes by heat-treatment of the nitrocellulose membrane before immunostaining would improve their detectability. One syntaxin antibody (clone 78.3) recognized only the 230 kDa band on untreated membranes (Fig. 1C), but identified both the 100 kDa band and (with markedly enhanced staining) the 230 kDa band after heat-treatment of the blotting membrane (Fig. 1D). Two different synaptobrevin 2 antibodies that detected only the monomeric antigen in untreated nitrocellulose membranes were able to detect slowly migrating bands only after heat-treatment of the blotting membrane: one antibody (clone 69.1) stained the higher, the other one (AB5856) the lower of the two bands that were detected by the antibody against SNAP-25 (Fig. 1E).

When films were exposed for extended periods of time (>1 hour) to capture the low intensity signals obtained with the antibodies against synaptobrevin 2, an additional band of about 55 kDa became visible that was also detected by the SNAP-25 antibody on untreated and heat-treated blotting membranes (Fig. 1E) and by the syntaxin1 antibody (clone 78.3, not shown).

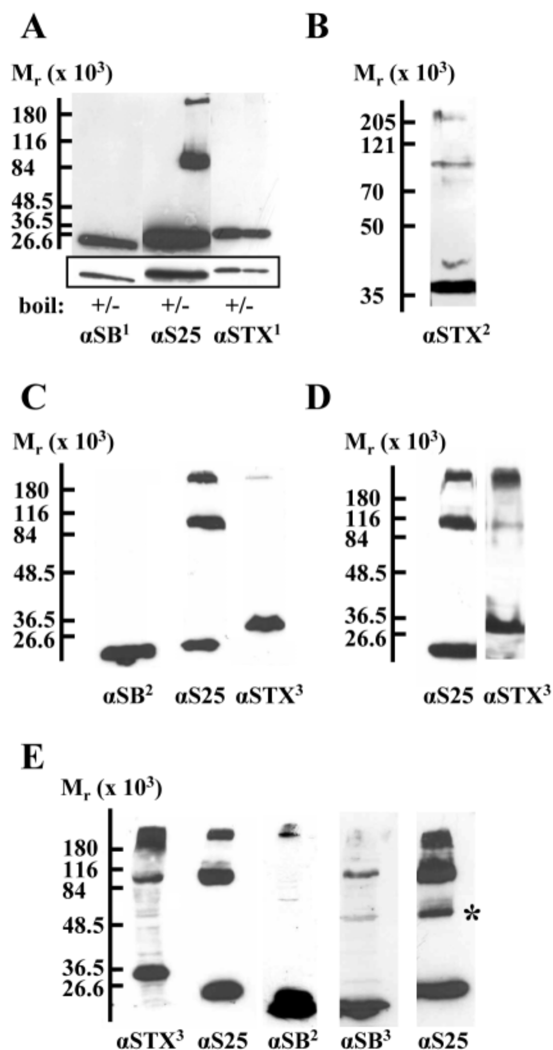

Fig. 1. Identification of SDS-resistant SNARE complexes in PC12 cell membrane extracts. SDS-membrane extracts were separated on SDS-PAGE, transferred to nitrocellulose membranes and were immunoblotted with polyclonal and monoclonal antibodies against SNARE. (A) Immunoblot of unboiled samples and samples that had been boiled before electrophoresis from a single membrane preparation using MAB333 (αSB[1]), MAB331 (αS25) and MAB336 (αSTX[1]). The insert at the bottom shows the anti-SNARE bands that correspond to the monomeric antigens from a shorter film-exposure of the nitrocellulose membrane. Of the three antibodies only MAB331 (antibody against SNAP-25) detects heat-sensitive SDS-resistant SNARE complexes. However, the amount of monomeric syntaxin and synaptobrevin is increased in boiled samples, suggesting that in the unboiled samples a fraction of these proteins is engaged in protein complexes where epitopes are inaccessible for the respective antibody. Immunoblots shown in B to E were performed with unboiled SDS-samples. (B) Syntaxin antibody clone HPC-1 (αSTX[2]) detects, besides the monomeric antigen at about 36 kDa, two high-molecular weight bands at positions, which correspond to the complex bands identified with MAB331 and shown in A (at ~230 and ~100 kDa). (C) Syntaxin-antibody clone 78.3 (αSTX[3]) recognizes only the 230 kDa protein band (with low staining intensity) which is also detected by MAB331 (αS25), whereas synaptobrevin antibody clone 69.1 (αSB[2]) fails to detect any slow migrating bands. (D) After heat-treatment of the nitrocellulose membrane, αSTX[3] recognizes both high-molecular weight bands that are also detected by MAB331 (αS25). (E) On heat-treated nitrocellulose membranes, synaptobrevin antibody clone 69.1 (αSB[2]) detects the 230 kDa band, whereas synaptobrevin antibody AB5856 (αSB[3]) recognizes only the 100 kDa protein band. Note that the samples separated in lanes 1-3 and lanes 4 and 5 (from left to right), were from the same membrane preparation. The preparation analysed in lanes 4 and 5 contained a third SDS-resistant band at about 55 kDa (asterisk). The recognition patterns of the antibodies were verified in at least three independent experiments.

These results demonstrate, on the one hand, that the complexes detected by the SNAP-25-antibody also contain synaptobrevin and syntaxin and, on the other hand, that no further SDS-resistant SNARE complexes are present in the PC12 cell membrane extracts, such as one with an epitope inaccessible for the SNAP-25 antibody.

Because the antibody against SNAP-25 (MAB331) detected the SDS-resistant SNARE complexes without the requirement for heat-treatment of the blotting membrane, we used primarily this antibody in all subsequent experiments to investigate the behavior of these two complexes under different conditions. By using this antibody, the lowest band (which presumably corresponds to monomeric SNAP-25) was detectable when less than 1 μg of membrane protein was loaded, whereas the higher bands were less abundant and required loading of ≥10 μg of total membrane protein (Fig. 2A). The 55 kDa band (Fig. 1E) was not routinely identified and was thus either absent from most of our preparations or present at levels below detection limits. To test whether the same SDS-resistant SNARE complexes exist before the isolation of PC12 membranes, we directly lysed intact cells in SDS-sample buffer. In blots with these samples the anti-SNAP-25-immunoreactive bands, of ~100 and 230 kDa, were stained, although higher total protein concentrations per lane were required for their detection (Fig. 2B). We will further refer to them as complex-100 and complex-230, respectively.

A property described for native SNARE complexes and for complexes formed from recombinant SNARE proteins is the temperature dependence of their SDS-resistance (Hao et al., 1997; Hu et al., 2002a). To test whether this was also true for SDS-resistant complexes in PC12 cells, we investigated the temperature-sensitivity of complex-100 and complex-230 in more detail (Fig. 3A). When PC12 cell proteins were incubated in SDS-containing sample buffer for 5 minutes at different temperatures, the amount of both complexes decreased with increasing temperatures albeit with clearly different thermal stabilities. At 65°C, complex-230 was reduced to 4% of the band obtained after incubation at 37°C, as determined by densitometry. However, at this temperature, complex-100 was just slightly reduced and a similar reduction was achieved only after an incubation at 82°C. Once the cells were lysed, SNARE complexes were also unstable at lower temperatures in the presence of SDS or lysis buffer. When lysates of PC12 cells were kept at 24°C instead of –20°C for 16 hours before protein preparation, the intensity of the bands of the two SNARE complexes decreased by 85% or 99.7% (complex-230) and 43.4% or 85.5% (complex-100), relative to the band intensities obtained with protein preparations of lysates kept at –20°C for the same time (Fig. 3B). Moreover, a disruption of SNARE complexes in the presence of SDS at

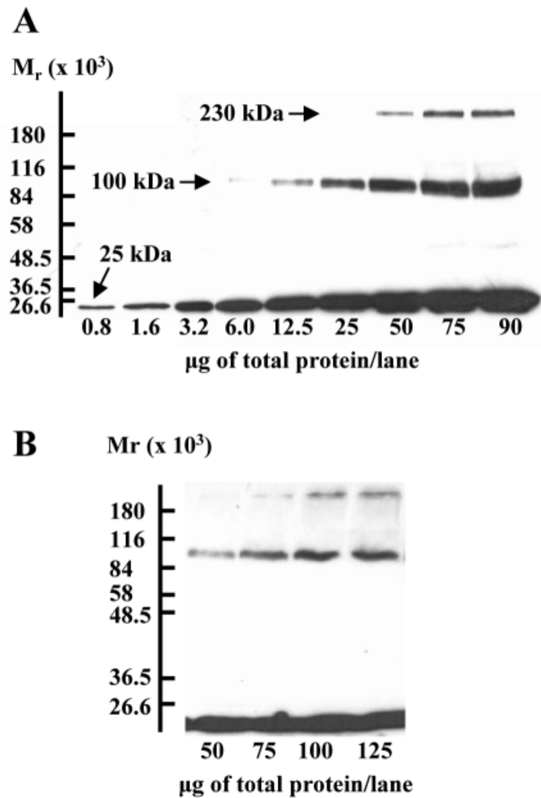

Fig. 2. SNARE-immunoreactive protein bands are identical in immunoblots of total PC12 cell extracts and in extracts of isolated PC12 cell membranes. (A) Increasing amounts (left to right) of total membrane protein extracted from PC12 cell membranes were loaded as indicated, separated on SDS-PAGE and transferred to a nitrocellulose membrane. SNAP-25-immunoreactive material was identified by anti-SNAP-25 (MAB331) antibody at a position characteristic for monomeric SNAP-25 and at ~100 and ~230 kDa, respectively. (B) Similar blot as shown in A for protein samples from whole PC12 cells directly lysed in SDS-sample buffer.

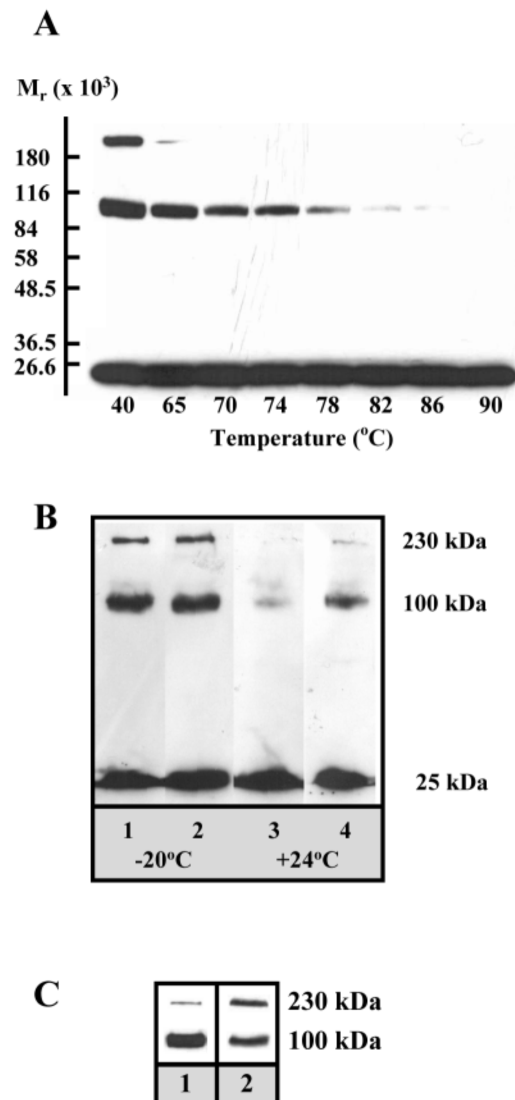

Fig. 3. Temperature dependence and variation of relative amounts of SDS-resistant SNARE complexes from PC12 cell membranes. (A) An anti-SNAP-25-immunoblot was performed on PC12 cell SDS-membrane-extracts that had been exposed to different temperatures (as indicated) for 5 minutes before separation by SDS-PAGE. (B) SNAP-25-immunoreactive bands of membrane extracts from cells lysates kept at –20°C (lanes 1, 2) or +24°C (lanes 3, 4) for 16 hours before protein preparation. (C) Comparison of 100 kDa and 230 kDa SNAP-25-immunoreactive bands from two independent preparations (lanes 1, 2) of PC12 cells.

room temperature (≤24°C) was evident from the loss of slowly migrating bands in samples not subjected to electrophoretic separation within 48 hours (not shown). Thus, the intensities of immunoreactive bands but not the band pattern detected by the antibody against SNAP-25 crucially depended on the protein preparation procedure.

Using a standard preparation procedure not only the absolute but also the relative amounts of complex-100 and complex-230 varied considerably between preparations. Typical ratios of complex-100 and complex-230 were about 10:1, but occasionally almost equal intensities of these two bands were observed (Fig. 3C). Importantly, the amounts and ratios of these complexes were constant when protein preparations were obtained from one set of PC12 cell cultures under identical experimental conditions. Therefore, it appeared possible to evaluate whether changes in SNARE complexes might occur under certain physiological conditions, such as secretion, by comparing the band intensities of complex-100 and complex-230 isolated from control and stimulated cells. We depolarized PC12 cells by exchanging the physiological buffer with buffer containing a higher K^+ concentration (Na^+ was reduced accordingly). Thereafter proteins were extracted from isolated PC12 membranes directly into SDS-sample buffer and immediately separated by electrophoresis, SDS-resistant SNARE complexes were analysed on a western blot. These experiments allowed us to address two important questions in current SNARE research, namely the occurrence and the fusion competence of SDS-resistant SNARE complexes in living cells: (1) because control cells and stimulated cells were processed in parallel using a standard preparation procedure (see Materials and Methods) any changes in the band intensities of complex-100 and complex-230 must be due to effects occurring in the intact cells before membrane

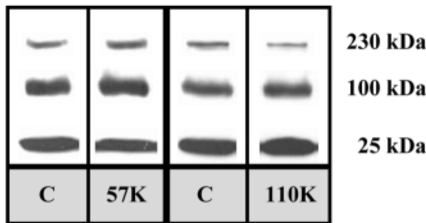

Fig. 4. Effect of K^+-stimulation on SNARE complexes. 230 kDa, 100 kDa and 25 kDa SNAP-25-immunoreactive bands are shown from samples extracted from unstimulated (control) cells (C) and from cells that were stimulated for 5 minutes with 57 mM K^+ or 110 mM K^+ (57K, 110K, respectively).

preparation. If the physiological conditions applied to intact PC12 cells could change the levels of the two SDS-resistant SNARE complexes, this would suggest that these complexes exist in vivo. (2) There is evidence that fusion-competent SNARE complexes form during vesicle priming (Banerjee et al., 1996b; Lonart and Sudhof, 2000; Wei et al., 2000; Xia et al., 2001) (reviewed in Rizo and Sudhof, 2002) and are disassembled by the ATPase NSF (Littleton et al., 2001; Weber et al., 2000) in a post-fusion step. Ca^{2+} per se does not cause disruption of SNARE complexes (Chen et al., 1999; Leveque et al., 2000; Hu et al., 2002a). According to this model the amount of fusion-competent complexes should decrease as a function of secretory activity. Moreover, if the two complexes perform identical functions, they should be affected by the stimulation of secretion in a similar manner.

Secretory activity was stimulated by exposing PC12 cells for 5 minutes to a series of K^+-concentrations ranging from 30 to 110 mM. This treatment consistently caused changes in the amounts of SDS-resistant SNARE complexes. However, a reduction of SDS-resistant complexes was observed only for complex-230 and required K^+-concentrations of ~80 mM or more. Lower concentrations of K^+ did not cause any reduction but instead an increase of SDS-resistant SNARE complexes was induced with 57 mM K^+. Typical experiments are shown in Fig. 4. Following a 5 minute incubation in 57 mM K^+, complex-230 appeared to be enhanced when compared with control cells incubated in 4 mM K^+. Complex-100, by contrast, remained largely unchanged (Fig. 4, left) Densitometric analysis revealed an increase to 148.0±22.7% for complex-230 (n=4), but no obvious change for complex-100 (106.8±10,8%, n=4). By contrast, when PC12 cells were incubated for 5 minutes in 110 mM K^+, complex-230 was reduced and complex-100, if anything, displayed a small increase (Fig. 4, right). On average, complex-230 was reduced to 49.3±13.6% by 110 mM K^+ (n=4), but complex-100 amounted to 114.4±6.1% (n=4) when compared with the respective control cells. Thus, depolarization of PC12 cells appears to be sufficient to cause changes in SDS-resistant SNARE complexes, but the two anti-SNAP-25 immunoreactive bands were differently affected. Furthermore, different intensities (moderate or strong) of depolarization had opposite effects.

Transmitter release is controlled by the concentration of intracellular free Ca^{2+}. If the reduction of complex-230 was indeed related to the secretory activity of the stimulated cells than this effect should be related to rises in cytoplasmic Ca^{2+}.

Therefore, we next studied the effect of high K^+ concentration under conditions that reduce Ca^{2+} influx (Fig. 5). In the presence of 1.5 mM extracellular Ca^{2+}, depolarization by 80 mM K^+ induced a decrease in the band of complex-230. However, when cells were depolarized by 80 mM K^+ in the presence of only 0.1 mM extracellular Ca^{2+}, no change in the levels of SDS-resistant SNARE complexes was observed. Likewise, when voltage-gated Ca^{2+} channels were blocked by the addition of 1 mM Cd^{2+}, the effects of 80 mM K^+ in 1.5 mM Ca^{2+} were prevented, and the intensities of the two complex bands were identical to those of cultures that had not been depolarized (Fig. 5A).

These data indicate that the elevation of intracellular free Ca^{2+} rather than the K^+-induced depolarization causes the loss of complex-230. This hypothesis was further tested in an alternative approach using permeabilized PC12 cells. PC12 cells were incubated in buffer containing digitonin in the presence of free Ca^{2+} at micromolar concentrations. Like the stimulation with high concentration of K^+, concentrations of 15 μM or more of free Ca^{2+} led to a reduction of complex-230 in permeabilized cells. In the experiments shown in Fig. 5B, the syntaxin antibody clone 78.3 was used on heat-treated blotting membranes to compare the effect of high K^+ stimulation of intact cells on SDS-resistant SNARE complexes with the differences in complex-band intensities, obtained when cells were permeabilized in the presence of 100 nm [~ the Ca^{2+} concentration usually found in resting PC12 cells (see below)] or 250 μm free Ca^{2+} (Fig. 8B below). In both cases, stimulation (depolarization and elevated intracellular free Ca^{2+}) reduced complex-230, but complex-100 was increased (intact cells) or remained unaffected (permeabilized cells). A similar experiment using the SNAP-25 antibody is shown in Fig. 5C for cells where complex-230 and complex-100 were present in almost equal amounts under control conditions. Here, the reduction of complex-230 was accompanied by a pronounced increase of complex-100 in cells permeabilized in the presence of 15 μM free Ca^{2+} and also in intact cells stimulated with high concentrations of K^+.

The results shown so far suggest that changes in SDS-resistant SNARE complexes occur when vesicle exocytosis is promoted by increases in intracellular Ca^{2+}. Accordingly, these changes should be reversible when intracellular Ca^{2+} is lowered again and exocytosis is thus reduced. Indeed, when PC12 cells were allowed to recover in 4 mM K^+ after depolarization by 80 mM K^+, complex-230 reappeared. During this cycle of depolarization and repolarisation, complex-100 was not diminished, but, if anything, displayed a slight increase upon repolarisation (Fig. 5A, lane D/R). In two equivalent experiments (Fig. 5C) after depolarization by 110 mM K^+ and subsequent repolarisation, the levels of complex-230 were not only restored but reached levels higher than those observed in samples from non-depolarized cells (136.1% and 155.4% of control, respectively). Under these experimental conditions, complex-100 was also increased when compared with samples from non-depolarized cultures (148.1% and 147.4% of control, respectively).

Taken together these data provide evidence that the reduction of complex-230 in stimulated PC12 cells is an entirely reversible, Ca^{2+}-dependent effect. Ca^{2+} not only triggers vesicle fusion but is also involved in priming of vesicles, albeit with different affinities to the molecular machineries responsible for these processes. In neuroendocrine

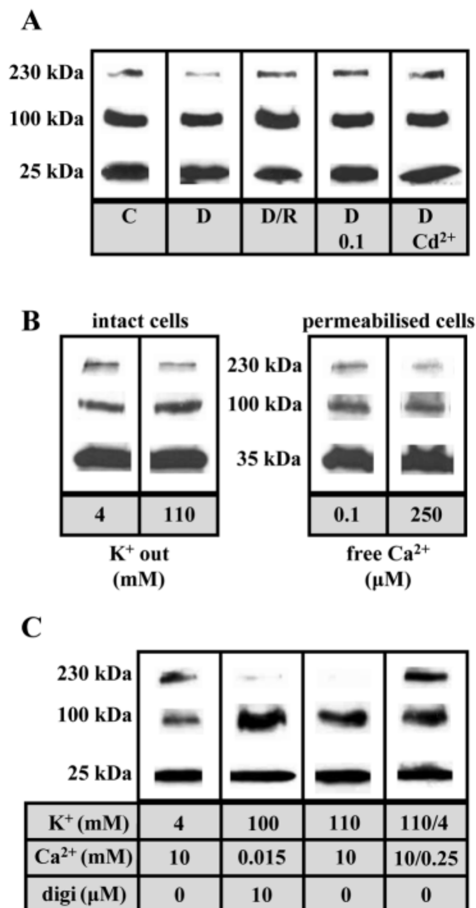

Fig. 5. Membrane-depolarization-induced effects on SNARE complexes in PC12 cells that were stimulated with high levels of K^+ require elevations of intracellular Ca^{2+}. (A) Effects of membrane depolarization and repolarization on SNARE complexes in cells at different extracellular Ca^{2+} concentrations or in the presence of 1 mM Cd^{2+}. 230 kDa and 100 kDa SNAP-25-immunoreactive bands from PC12 cell membrane extracts from cells treated for 5 minutes in the following way: (lane C) incubation in physiological buffer containing 1.5 mM Ca^{2+} (control), (lane D) stimulation with 80 mM K^+ buffer containing 1.5 mM Ca^{2+}, (lane D/R) stimulation with 80 mM K^+ buffer containing 1.5 mM Ca^{2+}, followed by a 45 minute incubation at 37°C in physiological buffer containing 0.1 mM Ca^{2+}, (lane D 0.1) stimulation with 80 mM K^+ buffer containing 0.1 mM Ca^{2+}, (lane D Cd^{2+}) stimulation with 80 mM K^+ buffer containing 1.5 mM Ca^{2+} and 1 mM Cd^{2+}. (B) The effect of membrane depolarization in PC12 cells with 110 mM K^+ on syntaxin (clone 78.3)-immunoreactive protein bands (intact cells, left panel) is shown together with a comparison of the intensities of protein bands recognized by the same antibody in PC12 cell membrane extracts from permeabilized cells exposed to 0.1 μM free Ca^{2+} and 250 μM free Ca^{2+} (right panel). Immunoblots were performed on heat-treated nitrocellulose membrane. Stimulation of intact cells with 110 mM K^+ for 10 minutes led to a decrease in the intensity of the 230 kDa band (62% of the band intensity obtained from an extract of non-depolarized control cells, left column) and to an increase in the intensity of the 100 kDa band (157% of control). Exposure of permeabilized cells to 250 μM free Ca^{2+} showed a reduced intensity of the 230 kDa band (to 63%) when compared with the intensity of the 230 kDa band when 100 nM free Ca^{2+} were present. The intensities of the 100 kDa band after stimulation of cells exposed to 0.1 μM and 250 μM free Ca^{2+} showed no difference (intensity in 250 μM Ca^{2+} was 99.6% of intensity in 0.1 μM Ca^{2+}). Notice that the 100 kDa and 230 kDa bands are from films exposed to the blotting membrane for different durations. (C) Effect of depolarization, repolarization and digitonin (digi)-permeabilization of PC12 cells. SNAP-25-immunoreactive protein bands obtained from membrane extracts from PC12 cells exposed for 10 minutes to the indicated conditions are compared with those of the control cells (4 mM K^+ and 10 mM Ca^{2+}) (bands on left side). Repolarization was for 45 minutes at 37°C (bands on right side).

cells submicromolar free Ca^{2+} was suggested to promote vesicle priming while causing only low rates of exocytosis, whereas free Ca^{2+} at micromolar concentrations is required for higher rates of vesicular release (Bittner and Holz, 1992; von Ruden and Neher, 1993; Smith et al., 1998; Voets, 2000) (reviewed in Rettig and Neher, 2002). We therefore hypothesized that the Ca^{2+} elevations evoked by moderate K^+-stimulation might be responsible for the increased amounts of SDS-resistant SNARE complexes (Fig. 4). To test this hypothesis, cells were permeabilized in the presence of 0.1-1 μM free Ca^{2+}. In these experiments, complex-230 displayed a concentration-dependent increase (Fig. 6A), and the intensity of the band obtained at 1 μM Ca^{2+} amounted to 225.7±47.6% (n=3) of that obtained at 0.1 μM Ca^2. By contrast, complex-100 remained rather unchanged, and at 1 μM Ca^{2+} the intensity of this band was 113.6±4.4% (n=3) of that found at 0.1 μM Ca^{2+} (Fig. 6B).

Undifferentiated PC12 cells are electrically non-excitable cells and thus depolarization fails to evoke action potentials (Dichter et al., 1977). Nevertheless, these cells posses voltage-activated Ca^{2+} channels and extracellular K^+ might evoke graded Ca^{2+} rises in a concentration dependent manner. From calculations based on changes in the K^+ equilibrium potential, 57 mM K^+ will depolarize cells about 20 mV less than 110 mM K^+. We speculated that the lack of reduction of complex-230 at concentrations below 80 mM might be owing to lower secretory activity of the cells.

To find out whether the different depolarizing conditions might cause distinct rates of vesicle exocytosis, we determined the amount of previously incorporated [^{3}H]noradrenaline (^{3}H-NA) that was released from PC12 cells when exposed to these K^+ concentrations. As expected, release of radioactivity was stimulated by both K^+ concentrations (Fig. 7A), but the amount released in response to 110 mM K^+ (29.9±3.1% of total ^{3}H-NA, n=3) was more than twofold higher than that released by 57 mM K^+ (13.6±1.1% of total ^{3}H-NA, n=3).

To obtain more detailed information on the time course of ^{3}H-NA release, similar experiments were performed with PC12 cells under continuous superfusion (Scholze et al., 2002). The superfusate was collected every 4 minutes and cells were exposed to 57 mM or 110 mM K^+ (Fig. 7B). Within the first 4 minutes of stimulation the net ^{3}H-NA release amounted to 28.2±4.3% for the stimulation with 110 mM K^+ (n=3) and to 16.0±1.7% for the stimulation with 57 mM K^+ (n=3). Between minutes 8 and 12, after the start of K^+-stimulation, the ^{3}H-NA release decreased to 7.8±1.8% (110 mM K^+) and 6.1±0.5% (57 mM K^+) of total ^{3}H-NA.

We next investigated whether the different rates of exocytosis induced by moderate and high K^+ stimulation might be caused by different increases in intracellular free Ca^{2+}. To reveal how differnt K^+ concentrations affect intracellular free

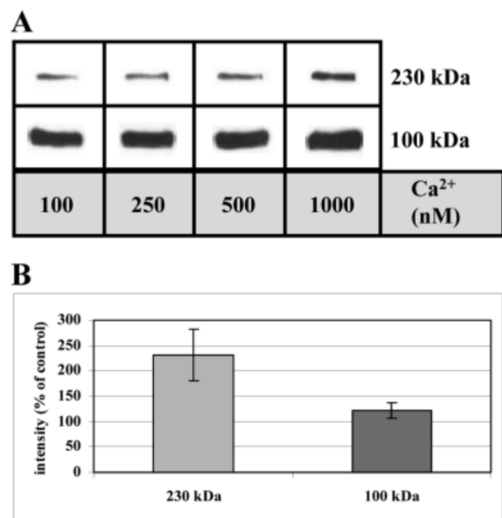

Fig. 6. Effect of submicromolar free Ca^{2+} on SNARE complexes in permeabilized PC12 cells. (A) SNAP-25-immunoreactive bands from samples extracted from PC12 cells that had been exposed to intracellular buffer containing 100, 250, 500 or 1000 nM free Ca^{2+} in the presence of 10 μM digitonin for 20 minutes before membrane preparation. (B) Comparison of the levels of SNAP-25-immunoreactive proteins in membrane extracts of cells permeabilized in the presence of 100 nM and 1000 nM free Ca^{2+}. Data are from three independent experiments. Levels of the 230 kDa band (light grey bar) and the 100 kDa band (dark grey bar) observed in the presence of 1000 nM free Ca^{2+} are shown as percentage (± s.d.) of the signals obtained with 100 nM free Ca^{2+}.

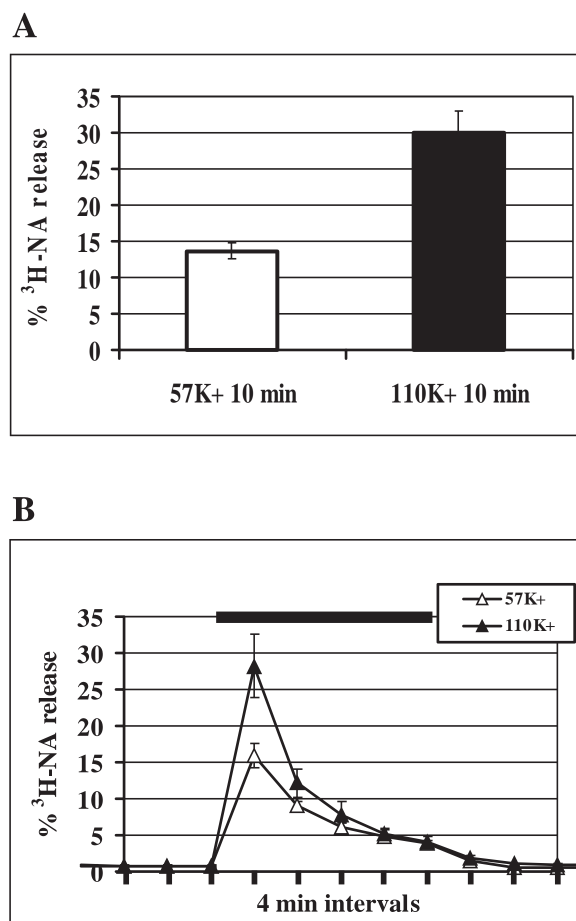

Fig. 7. Comparison of [3H]noradrenaline (^{3}H-NA) release evoked by 57 mM and 110 mM K^+-depolarization. (A) ^{3}H-NA release evoked within 10 minutes of stimulation with 57 mM K^+ or 110 mM K^+. The bars show the average response (± s.d.) of three measurements in % of total radioactivity. (B) ^{3}H-NA release induced within 20 minutes (black horizontal bar) of stimulation with 57 mM K^+ and 110 mM K^+ in superfused PC12 cells. Symbols show the average radioactivity (± s.d.) in superfusates collected every 4 minutes. Values were corrected for basal release, which was determined 12 minutes before membrane depolarization with K^+.

Ca^{2+}, PC12 cells were loaded with fura-2 acetoxymethyl ester, and changes in the fluorescence at 340 and 380 nm excitation wavelengths were determined.

The basal free Ca^{2+} concentration amounted to 111±29 nM (n=6) (8-16 cells per experiment). Both, 57 mM K^+ and 110 mM K^+ induced elevations of intracellular free Ca^{2+}, but these increases clearly differed in extent and duration (Fig. 8A). Ca^{2+} rises induced by 57 mM K^+ were sustained and on average reached a concentration of 503±105 nM (n=4) in the first minute. After 12 minutes, this rise in Ca^{2+} had slightly decreased to 431±85 nM (Fig. 8B). Compared with this, elevated Ca^{2+} levels evoked by 110 mM K^+ were, although considerably higher, transient, and decreased in the presence of elevated K^+ to levels closer to those evoked by 57 mM K^+ [peak at 3171±167 nM immediately after stimulation, 648±161 nM (n=3) 12 minutes after stimulation (Fig. 8B)].

Taken together, the depolarization of PC12 cells with moderate (57 mM) or high (110 mM) K^+ concentrations caused medium or large increases in intracellular Ca^{2+} and medium or high rates of vesicle exocytosis, respectively. Furthermore, the responses to 57 mM and 110 mM K^+ showed different kinetics: ^{3}H-NA release was transient in both cases, but the rise in Ca^{2+} was sustained for the duration of measurement when stimulated by 57 mM K^+.

This prompted us to investigate whether the opposing effects of these two K^+ concentrations on SDS-resistant SNARE complexes might also change with time. When the incubation of PC12 cells in 57 mM K^+ was prolonged from 5 to 10 minutes, the initial increased intensity of complex-230 was reduced to a mean intensity of 79.7±8.5% (n=3) of control (Fig. 9A and B). Complex-100, by contrast, remained rather stable and amounted to 91.8±17.4% (n=3)of control after 10 minutes in 57 mM K^+ (Fig. 9A and B). Prolonged incubation time (10 minutes) in 110 mM K^+ further decreased the amount of complex-230 to a mean intensity of 24.9±37.6% (n=3) of control. By contrast, the initial slight increase in intensity of complex-100 after 5 minutes rose further and amounted to 122.9±25.5% (n=3) of control after 10 minutes in 110 mM K^+ (Fig. 9B). Thus the increased intensity of complex-230 appears to be a transient phenomenon, only observed during mild depolarization and moderate increases in intracellular Ca^{2+}.

Discussion

Heat-sensitive SDS-resistant SNARE complexes consisting of the exocytotic proteins syntaxin, SNAP-25 and synaptobrevin

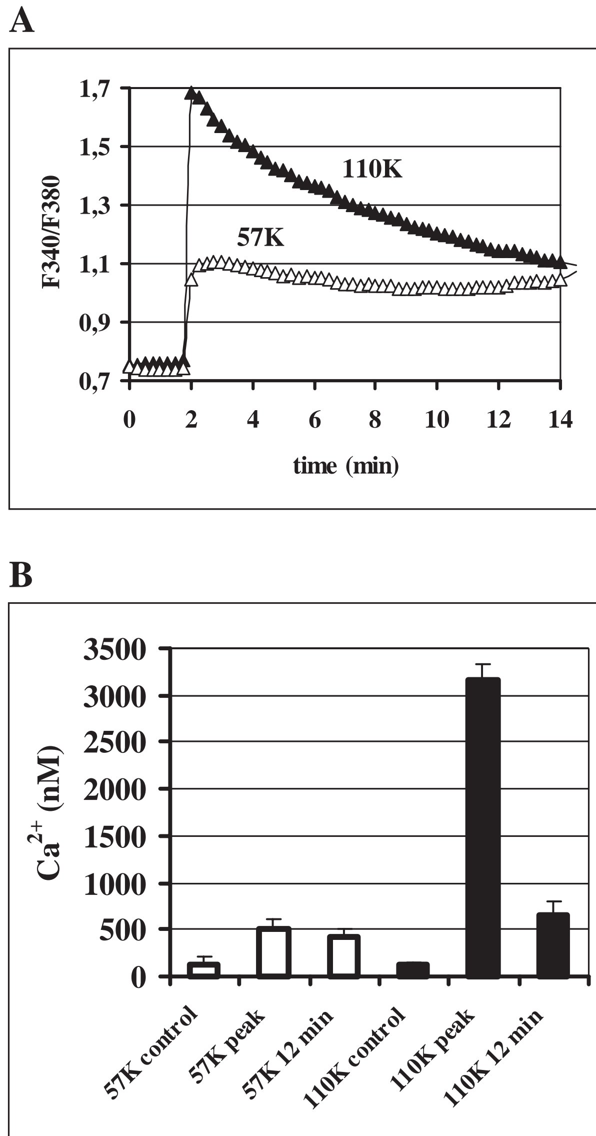

Fig. 8. Comparison of Ca^{2+} elevations evoked by membrane depolarization with 57 mM and 110 mM K^+. (A) Averaged fura-2 fluorescence ratios (F340/F380) measured in single PC12 cells stimulated with 57 mM K^+ (white triangles) or with 110 mM K^+ (black triangles). Fluorescence images were acquired every 15 seconds. Superfusion was switched from physiological buffer (containing 4 mM K^+) to high K^+ depolarization buffer at 1.85 minutes. The symbols indicate the mean fluorescence ratio of 16 (57 mM K^+) and 14 cells (110 mM K^+). (B) Analysis of Ca^{2+} responses induced by 57 mM K^+ and 110 mM K^+ from experiments such as the one shown in A. The bars show the average response (± s.d.) of 4 (57 mM K^+) and 3 (110 mM K^+) experiments (7-16 cells per experiment).

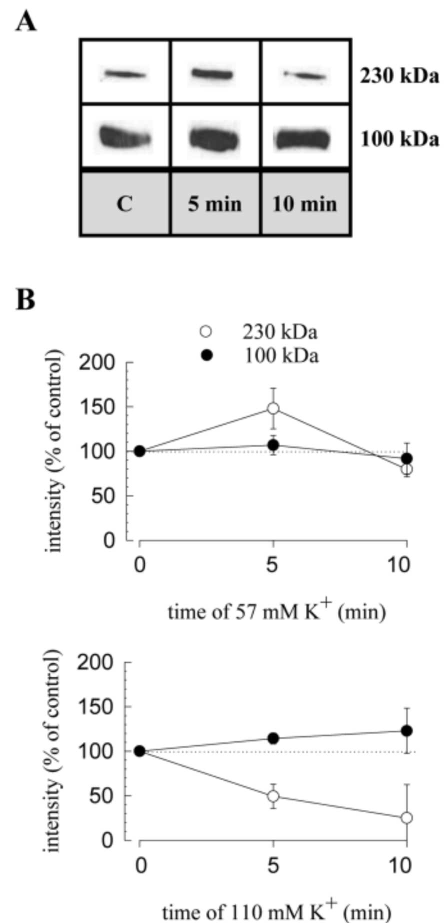

Fig. 9. Comparison of the effects of short- and long-lasting K^+-depolarizations on SNARE complexes. (A) SNAP-25-immunoreactive bands from membrane extracts of PC12 cells that were exposed to normal extracellular buffer (control, C) or to 57 mM K^+ buffer for 5 or 10 minutes before membrane preparation. (B) Densitometric analysis of western blots with samples obtained after 5 or 10 minute depolarization with K^+ (top graph, 57 mM K^+; bottom graph, 110 mM K^+). Averaged values (± s.d.) for the 100 kDa and 230 kDa bands from three to four experiments are shown as percentage of control.

were found in protein extracts from a large variety of preparations, including recombinant SNARE proteins and membrane extracts of neuronal (Hayashi et al., 1994; Lonart and Sudhof, 2000; Matveeva et al., 2003) and neuroendocrine cells (Lawrence and Dolly, 2002; Banerjee et al., 1996a; Chen et al., 1999). These complexes are thought to play a crucial role in regulated exocytosis, probably by bringing vesicular and target membranes into close contact. However, clear evidence for their exact mode of action is lacking. SNARE complexes might represent the minimal fusion machinery, might alternatively modulate the Ca^{2+}-sensitivity of the fusion process or might provide a platform for the assembly of a more extensive fusion machinery (Duman and Forte, 2003). Adding to the controversies, SDS-resistant SNARE complexes often exist in various forms with different electrophoretic mobilities that give rise to multiple anti-SNARE-immunoreactive bands on western blots. The structural basis for these multiple forms of SNARE complexes is unknown. It also remains unclear whether multiple complexes do actually exist in vivo or form only during the process of biochemical analysis. Accordingly, the question whether only one or several, all or none of the SDS-resistant complexes represent fusion-competent SNARE structures remains unanswered.

In PC12 cells we found two populations of SDS-resistant SNARE complexes. This is in contrast to the non-neoplastic

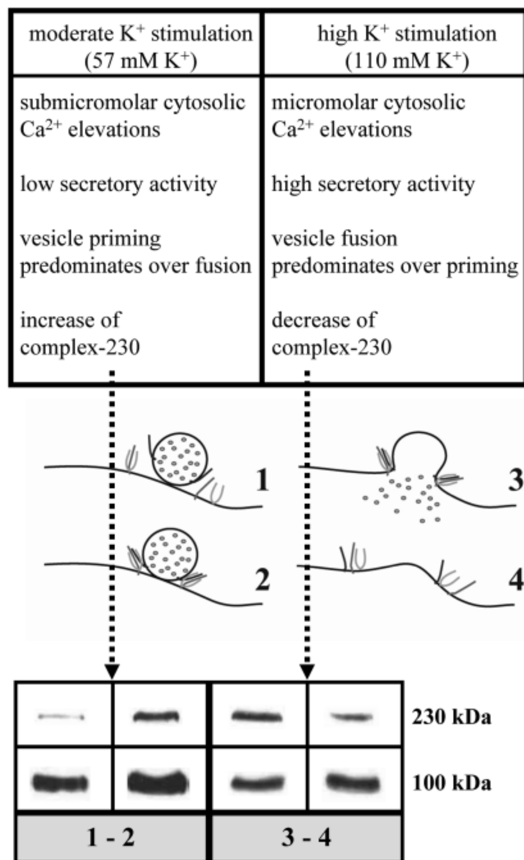

Fig. 10. The model describes how moderate and intense stimulation of exocytosis might affect SDS-resistant SNARE complexes in neuroendocrine cells. (Left) Ca^{2+} elevations to submicromolar concentrations (e.g. by 57 mM K^+) stimulate vesicle priming and support low rates of vesicle exocytosis (left drawing: (1) unprimed vesicle, (2) primed vesicle). As a consequence, the amount of fusion-competent SDS-resistant SNARE complexes is increased. This is illustrated in the lower panel showing cells permeabilized in the presence of 100 nM (1) and 1000 nM (2) free Ca^{2+}. (Right) Ca^{2+} elevations to micromolar concentrations (e.g. by 110 mM K^+) support vesicle priming and high rates of vesicle exocytosis [right drawing, (3)]. The immediate fusion of primed vesicles leads to a reduction of fusion-competent SDS-resistant SNARE complexes via a post-fusion action of NSF [right drawing, (4)] [shown in the lower panel for a 5 minute stimulation of intact cells with 110 mM K^+ (4) compared with SNAP-25-immunoreactive protein bands obtained from non-depolarized control cells (3)].

counterpart of PC12 cells, chromaffin cells. Using a two-step electrophoresis protocol, Lawrence and Dolly showed that multiple SDS-resistant SNARE complexes exist in chromaffin cells, but that only a selection of these can be identified using a conventional one-dimensional electrophoresis procedure. Hence, epitopes of SNARE proteins that are recognized by antibodies might be hidden within these complexes (Lawrence and Dolly, 2002). However, the observations described in this article show that only two SDS-resistant SNARE complexes exist in PC12 cells: (1) several of the SNARE antibodies that were used in this study detected SNARE complexes only after heat-treating the membrane before using it in a western blot. These antibodies (antibody against syntaxin 1 clone 78.3, antibody against synaptobrevin 2 clone 69.1, antibody against synaptobrevin 2 AB5856) have their epitopes in the N-terminal part of the respective SNARE proteins, a region that is engaged in coiled coil formation. Heat-treatment in the presence of SDS causes irreversible disassembly of the complexes. Thus, the failure of the antibodies to detect complexes before such a treatment is most likely attributable to hidden epitopes. However, with all antibodies used (including MAB331, which detected both complexes on untreated membranes), only complex-230 and complex-100 were detected after heat-treatment and no other protein bands became visible. (2) We also used a syntaxin antibody (clone HPC-1) that has its antigenic epitope in the extracellular C-terminal part of the protein. This part of the protein cannot be engaged in SNARE complex formation and the epitope should thus be accessible. Indeed, this antibody recognized both, complex-230 and complex-100, on untreated blotting membranes. However, the signal intensities were very low and thus this antibody was not further used in the present study. (3) We tested the two-step protocol of Lawrence and Dolly (Lawrence and Dolly, 2002) on PC12 cell samples, but MAB331 detected SNAP-25 only at positions corresponding to complex-100 and complex-230 (data not shown).

Considering their close relation, the observation that PC12 cells and chromaffin cells differ in their number of SDS-resistant SNARE complexes is surprising. An earlier study on chromaffin cells revealed the presence of multiple SDS-resistant SNARE complexes only in a lysosomal fraction (Hohne-Zell and Gratzl, 1996). It might therefore be that, variations in the purification protocols used by us and by Lawrence and Dolly (Lawrence and Dolly, 2002) lead to different contamination by sources other than the plasmalemma, which in turn might be the basis for the diverging results.

Comparison of the staining patterns of different SNARE antibodies in western blots of untreated and heat-treated nitrocellulose membranes revealed clear cut differences between the slow and the fast migrating SNARE complexes in PC12 cells. Syntaxin antibody clone 78.3 detected only complex-230 before heat-treatment but both complexes after heat-treatment of the nitrocellulose membrane. Two synaptobrevin 2 antibodies detected either complex-230 or complex-100 after heat-treatment and recognized only the monomer on untreated blotting membranes. Presumably, complex disassembly is only partial after heat-treatment thus exposing the respective epitope only in one but not in the other complex. Alternatively, nonoverlapping cross-reactivities of these antibodies against synaptobrevin might enable the detection of synaptobrevin isoforms, other than synaptobrevin 2, that might be present in only one complex. In any case, these data indicate that complex-230 and complex-100 can be distinguished by structural differences, which is also demonstrated by their differing thermal stabilities.

The above described structural differences are paralleled by functional differences. Under conditions that enhance vesicle exocytosis, only complex-230, but not complex-100, displayed unequivocal changes. However, while increases or decreases of complex-230 depended on the extent of intracellular Ca^{2+} rises (see below), levels of complex-100 remained unchanged or were only slightly increased. A reduction in complex-230 was not necessarily accompanied by an increase in complex-100,

and an increase in complex-230 was never accompanied by a decrease in complex-100. It therefore appears unlikely that complex-230 is directly transformed into complex-100 or vice versa in the course of vesicle fusion. Accordingly, our data indicate that the two SDS-resistant SNARE complexes in PC12 cells not only differ in structural but also in functional aspects.

Increases and decreases in complex-230 during conditions of enhanced vesicle exocytosis were related to different rises in intracellular Ca^{2+} as evidenced by the following findings: (1) depolarization of PC12 cells by 80 mM K^+ caused a decrease in complex-230. This was was prevented when extracellular Ca^{2+} levels were reduced to 0.1 μM and when voltage-gated Ca^{2+} channels were blocked by Cd^{2+}. (2) Stimulation of PC12 cells with 57 mM K^+ increased intracellular Ca^{2+} levels (to ~0.5 μM) and increased the level of complex-230, whereas stimulation with 100 mM K^+ resulted in an increase of intracellular Ca^{2+} (to ~3 μM) and reduced the level of complex-230. The average intracellular Ca^{2+} concentration determined by fura-2 does not reflect the Ca^{2+} concentration in the submembrane compartment (Marsault et al., 1997) where vesicle fusion occurs. Nevertheless, these results show that different increases in intracellular Ca^{2+} can have opposite effects. (3) Exposure of permeabilized PC12 cells to 1 μM Ca^{2+} caused increases in complex-230; exposure to ≥15 μM caused decreases. Because the permeabilization of PC12 cells that were kept in 0.5 μM Ca^{2+} did not significantly increase complex-230 (+16.5%, see Fig. 6A), one can assume that the submembraneous Ca^{2+} concentration, achieved by stimulation with 57 mM K^+, was higher than the 0.5 μM measured by fura-2 microfluorometry.

In neuroendocrine cells, submicromolar Ca^{2+} levels evoke only little vesicle release but promote vesicle priming, with Ca^{2+} concentrations between 0.5 and 1 μM having the maximum effect (Bittner and Holz, 1992; von Ruden and Neher, 1993; Smith et al., 1998; Voets, 2000) (reviewed in Rettig and Neher, 2002). Micromolar levels of Ca^{2+}, however, trigger extensive vesicle exocytosis (Ahnert-Hilger et al., 1987; Bittner and Holz, 1992). Hence, increases in complex-230 are observed under conditions that favour vesicle priming, whereas decreases are observed under conditions that favour vesicle exocytosis (Fig.10). When PC12 cells were depolarized with K^+ over a long period, ^{3}H-NA release decreased but never stopped completely. At the same time, low amounts of complex-230 always remained. Even when there was an apparent loss of all complex-230 (Fig. 5C) residual complex-230 was seen on western blots when the sample volume was doubled (data not shown). Release of ^{3}H-NA induced by moderate K^+ concentrations also decreased with time, despite a sustained Ca^{2+} rise. Likewise transient was, the initial increase in complex-230 under these conditions, and between 5 and 10 minutes of stimulation a decrease in complex-230 was observed. The Ca^{2+}-dependent priming of vesicles enhances the Ca^{2+}-sensitivity of the pool of vesicles that is ready to be released (Voets, 2000). Priming and fusion of this particular vesicle pool might therefore occur in direct succession owing to very similar Ca^{2+} requirements. Accordingly, the loss of vesicles that are primed and fused in a Ca^{2+}-dependent manner might be responsible for the late reduction of compex-230 during membrane depolarization by moderate K^+ concentrations.

Our results suggest that Ca^{2+}-dependent vesicle priming is accompanied by SNARE complex formation. Several mechanisms might underlie the Ca^{2+}-induced SNARE complex assembly. For instance, Ca^{2+} might prevent the binding of regulatory proteins that inhibit complex assembly to uncomplexed SNARE proteins (Bean et al., 1997), e.g. via PKC-mediated phosphorylation (Fujita et al., 1996). Alternatively, synaptobrevin can be embedded C-terminally in the vesicular membrane (a conformation incompatible with SNARE complex formation) and may be freed by the action of Ca^{2+}/calmodulin (Quetglas et al., 2002; Kweon et al., 2003).

Our results do not exclude a role of SDS-sensitive SNARE complexes in vesicle exocytosis, but they indicate the presence of SDS-resistant SNARE complexes in living PC12 cells and of a slowly migrating complex species that might be involved in transmitter release in neuroendocrine cells. These conclusions are in line with recent reports, which show that complexin promotes the formation of slowly migrating SNARE complexes (Hu et al., 2002b) and is required for Ca^{2+}-regulated exocytosis (Reim et al., 2001). These findings taken together with our results suggest that higher order complexes represent the fusion competent SNARE structures. Such structures have been largely neglected in previous studies on the function of the SNARE fusion machinery and should be looked at carefully in future work in the field. This notion is supported by recent evidence that oligomeric SNARE structures form at fusion sites (Cho et al., 2002) and by mathematical modelling, which suggests that cooperation of at least three SNARE complexes is needed to mediate membrane fusion (Hua and Scheller, 2001; Yersin et al., 2003). The complex-230 observed in PC12 cells might represent such cooperative SNARE structure.

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