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Synaptic proteins and SNARE complexes are localized in lipid rafts from rat brain synaptosomes☆

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Abstract

The biochemical characterization of the SNARE proteins present in lipid microdomains, also known as "lipid rafts," has been addressed in earlier studies, with conflicting data from different laboratories. In this study, we use rat brain synaptosomes as a model with which to examine the presence of proteins involved in exocytosis in detergent-resistant membranes (DRM), also known as 'lipid rafts.' By means of buoyancy analysis in sucrose gradients of Triton X-100-solubilized synaptosomes, we identified a pool of SNARE proteins (SNAP 25, syntaxin 1, and synaptobrevin2/VAMP2) significantly associated with DRM. Furthermore, Munc18, synaptophysin, and high amounts of the isoforms I and II of synaptotagmin were also found in DRM. In addition, SDS-resistant and temperature-dependent SNARE complexes were also detected in DRM. Treatment of synaptosomes with methyl-β-cyclodextrin resulted in persistence of the proteins present in the DRM isolated using Triton X-100, whilst strongly impairing calcium-dependent glutamate release. The results from the present work show that lipid microdomains are sites where SNARE proteins and complexes are actually present, as well as important elements in the control of regulated exocytosis.

Keywords: Exocytosis; Membrane microdomains; SNARE proteins; Synaptic vesicles; Lipid rafts

There has been much interest recently in the role played by the lipid components of membranes in various cell processes, including exocytosis [1]. The membrane microdomains referred to as 'lipid rafts' [2] have received much attention as potential regulators and organizing centers for signal transduction and membrane trafficking pathways [3,4]. Lipid rafts are membrane microdomains that are enriched in cholesterol, sphingolipids, and GPI-anchored proteins, and were initially characterized biochemically by their relative insolubility in cold Triton X-100 [5]. Although Triton X-100 is the detergent most widely used for the purification of rafts, other detergents have also been used for this purpose, including Chaps, Lubrol WX, Brij96, and Triton X-114 [6]. This new array of detergents generates other types of DRM that differ from the classically described 'Triton rafts,' with different lipidic composition and characteristics. Although lipid rafts have been proposed to perform a key role in the sorting of certain membrane proteins in polarized epithelial cells [7], little is known about the involvement of these microdomains in the organization of the exocytotic machinery in specialized secretory cells.

Vesicular trafficking is essential for a variety of processes in eukaryotic cells, including maintenance of distinct subcellular compartments, protein and hormone

☆ *Abbreviations:* DRM, detergent-resistant membranes; GPI, glycosylphosphatidylinositol; MCDX, methyl-β-cyclodextrin; NSF, *N*-ethylmaleimide-sensitive factor; SV, synaptic vesicles; SV2, synaptic vesicle protein 2; SNAP 25, synaptosomal-associated protein of 25 kDa; SNARE, soluble NSF-attachment protein receptor; TLC, thin-layer chromatography; VAMP, vesicle-associated membrane protein.

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secretion, egg fertilization, and neurotransmitter release. Soluble *N*-ethylmaleimide-sensitive factor (NSF)-attachment protein receptor (SNARE) proteins are essential components of the vesicular exocytotic machinery. Three SNARE proteins are involved in synaptic vesicle (SV) exocytosis: the plasma membrane-associated proteins syntaxin 1 and synaptosomal-associated protein of 25 kDa (SNAP 25), and the membrane synaptic vesicle protein synaptobrevin, also referred to as vesicle-associated membrane protein (VAMP). Other conserved proteins include the ATPase NSF and its adaptor soluble NSF-attachment protein (SNAP), the Rab class of small G proteins and their effectors, the synaptotagmin family, and the Munc18 family (mammalian homolog of the yeast Sec1 protein) (see [8] for a review). In mammals, the synaptotagmin family contains 12 members [9]; isoforms I and II are membrane-associated proteins that interact with the SNARE complex independently of Ca^{2+}, although the interaction is enhanced by Ca^{2+} addition [10]. Many other factors that interact with SNAREs have been characterized, such as complexin, VAP33, and synaptophysin (see [11] for a review). These molecules may either be recruited from precursor pools that diffuse freely in the plane of the membrane or they may be pre-assembled in clusters before contact between the fusing membranes [12]. For instance, more than a dozen proteins have been identified that bind specifically to syntaxin 1, suggesting the presence of complicated networks of interrelated protein–protein interactions [13]. It is not clear whether such complexes are evenly distributed over the membrane surface or how they affect vesicle docking and fusing. Moreover, differences between the localization of syntaxin 1 and that of SNAP 25 in lipid rafts obtained with Triton X-100 have been described in PC12 cells [14,15]. Here we examine the distribution of proteins involved in exocytosis in segregated membrane domains in neural tissue.

Experimental methods

Antibodies. Monoclonal antibodies against GAP-43, synaptophysin, and syntaxin 1a/b (HPC-1) were from Sigma (St. Louis, MO, USA). The OX-7 monoclonal antibody against Thy-1 and polyclonal antibodies against synaptotagmins I and II were a gift from G. Schiavo (Cancer Research UK, London). Monoclonal antibodies against synaptotagmin I (clone 41.1) and VAMP2 were from Synaptic Systems (Goettingen, Germany). Monoclonal anti-rab3a was a gift from R. Jahn (University of Goettingen, Germany). A monoclonal antibody against munc18 (clone 31) was purchased from Transduction Laboratories (Lexington, USA). Anti-SNAP 25 (SMI81) was from Stenberger-Meyer (Jarretsville, MD). Anti-synapsin 1 was from Chemicon (Temecula, USA). Monoclonal anti-SV2 was a gift from K. Buckley (University of Liverpool). The anti-rab5 polyclonal antibody was from Stressgen (Victoria, Canada).

Preparation of synaptosomes from rat brain. All experiments were performed with a crude synaptosomal fraction (P2) prepared from 4- to 6-week-old Sprague–Dawley rat brains, as described previously [16], with slight modifications. The whole brain was homogenized in 40 vol. (w/v) phosphate buffer (pH 7.4) supplemented with 0.32 M sucrose. Homogenization was performed with 12 strokes (900 rev/min) of a Potter homogenizer with a Teflon pestle (0.1 ± 0.15 mm clearance). The homogenate was centrifuged at 1000*g* for 5 min at 4 °C. The supernatant was then centrifuged at 12,000*g* for 20 min. The crude synaptosomal pellet obtained from one brain was gently resuspended in 10 ml sodium buffer containing 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 20 mM Hepes/NaOH, and 10 mM glucose, and the pH was adjusted to 7.4. Protein concentration was determined according to the Bradford protocol.

Isolation of DRM. Synaptosomal aliquots (5 mg total protein) were solubilized with 2 ml cold sodium buffer containing 1% Triton X-100 by end-over-end mixing (30 min, 4 °C). The extract was then adjusted with 41% sucrose, and overlaid with 8 ml of 35% sucrose in sodium buffer and then 2.5 ml of 16% sucrose in sodium buffer. DRM fractions were isolated by ultracentrifugation at 35,000 rpm for 18 h at 4 °C [17]. Then, 12 fractions of 1 ml were collected. For saponin treatment, the protocol was the same as above, except that synaptosomes were solubilized in 0.5% Triton X-100 + 0.5% saponin. When indicated, synaptosomes were pretreated with methyl-β-cyclodextrin (25 or 50 mM) for 15 min, in order to deplete cholesterol from the plasma membrane.

Electrophoresis and Western blotting. Samples of each fraction from the sucrose gradient were analyzed by SDS–PAGE followed by Western blot. The separated proteins were then transferred to a Protran nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), using a Mini TransBlot Cell 3 (Bio-Rad, CA, USA) at 100 V for 1 h. The blotting buffer used contained 25 mM Tris, 200 mM glycine, and 10 % (v/v) methanol. The membrane filters were blocked for 1 h with Tris-buffered saline supplemented with 0.1% Tween 20 and 5% (w/v) defatted powdered milk. Then, the membranes were incubated overnight with the corresponding antibody diluted in blocking buffer. Next, the membrane filters were incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody diluted in blocking buffer. Several washes with Tris-buffered saline/0.1% Tween 20 were performed between each of the steps. Blots were developed using ECL detection reagents from Amersham–Pharmacia Biotech (Little Chalfont, UK) and visualized using a GeneGnome chemiluminescence detection system coupled to a CCD camera (Syngene, Cambridge, UK). When required, quantification of the bands was performed using the GeneTools program (Syngene, UK).

Cholesterol determination. Cholesterol was enzymatically determined in a Cobas-Mira automatic analyzer using a commercial reactive mixture specific for total cholesterol quantification (BioSystems, Spain) containing 35 mM Pipes, pH 7.0, 0.5 mM sodium cholate, 28 mM phenol, 0.2 U/ml cholesterol esterase, 0.1 U/ml cholesterol oxidase, 0.8 U/ml peroxidase, and 0.5 mM 4-aminoantipyrine. The method used is based on the spectrophotometric determination of quinonimine, a product of two consecutive reactions of the enzymes cholesterol oxidase and peroxidase. Cholesterol determination was performed by means of thin-layer chromatography (TLC) as follows: aliquots of 100 μl from each sucrose gradient fraction were mixed with 480 μl of a chloroform/methanol mixture (1:2). Subsequently, 200 μl of chloroform and 200 μl of water were added. After shaking the mixture, tubes were centrifuged (1000 rpm, 5 min), and the organic phase was extracted, evaporated, and resuspended in 20 μl chloroform/methanol (4:1). The lipid extract was then resolved on silica high-performance TLC plates using petroleum ether/diethyl ether/acetic acid (79:19:2). Staining of the lipids was performed using phosphomolybdic acid. Purified cholesterol was used as a standard.

Glutamate release. Glutamate release studies were performed as described previously [18]. Briefly, aliquots of the P2 fraction were resuspended in 500 μl of prewarmed sodium buffer and incubated at 37 °C. Synaptosomal suspensions were transferred to a cuvette containing 1.5 ml of the same buffer containing 1 mM NADP, 100 U glutamate dehydrogenase (Sigma, Poole Dorset, UK), and 1.3 mM $CaCl_2$ in a thermoequilibrated chamber. Glutamate release was

monitored with the aid of a spectrofluorophotometer (Shimadzu RF5001PC). Exocytosis was triggered by addition of KCl (final concentration, 50 mM) or ionomycin (final concentration, 10 μM). L-Glutamate (5 nmol) was added at the end of every determination, as an internal standard for calibration.

Results

Analysis of DRM isolated with Triton X-100 in rat brain synaptosome preparations

In the present work, a well-established protocol was used to purify lipid rafts from rat brain synaptosomes. After isolating the crude synaptosomal fraction, Triton X-100-resistant membranes were purified on the basis of flotation after ultracentrifugation in a discontinuous sucrose gradient (41%/36%/16%). Cytosolic proteins and solubilized membrane proteins remain in the 41% sucrose layer, whilst insoluble lipid rafts have a lower buoyant density and float in the 16% sucrose layer. Twelve fractions were collected, fractions 1–12 corresponding to divisions from the top to the bottom of the tube. In order to initially characterize the sucrose profile of rat brain synaptosomes, the protein and cholesterol concentrations were determined. Approximately 75% of protein extracted with 1% Triton X-100 was present in fractions 9–12, which correspond to the soluble fractions (Fig. 1A). Cholesterol was mainly present in insoluble fractions 1–3, showing that the majority remains included in DRM after solubilization with Triton X-100 (Figs. 1A and C), with only a small amount being extracted. In order to demonstrate the efficient separation of 'lipid rafts' in the sucrose gradient, Thy-1, a GPI-anchored neuronal raft protein, and GAP-43, a palmitoylated protein of the neuronal membrane, were immunodetected in the gradient fractions (Fig. 1B). Thy-1 was exclusively present in insoluble fractions 1–4, whereas GAP-43 was mainly present in the soluble fractions (Fig. 1B).

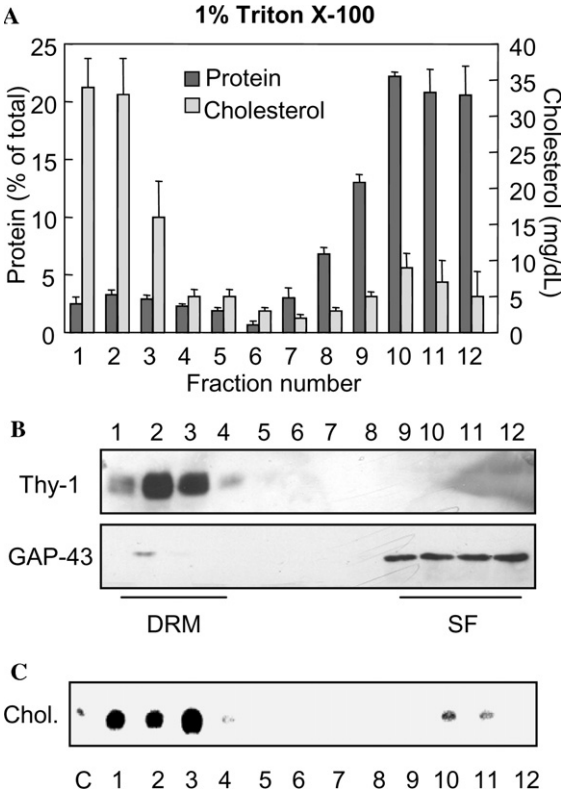

Fig. 1. Characterization of DRM fractions isolated from rat brain synaptosomes. (A) The synaptosome-enriched fraction was solubilized in cold 1% Triton X-100 and fractionated on a discontinuous sucrose gradient, as described under Experimental methods. The protein content of the gradient fractions (fractions 1–12 correspond to equal divisions from the top to the bottom of the tube) is shown as a percentage of the total protein in the gradient, and the amount of cholesterol is expressed in concentration units (mg of cholesterol dL^{-1}). (B) Equal volumes of the gradient fractions from (A) were separated by SDS–PAGE and transferred to nitrocellulose for immunoblot analysis using antibodies specific for Thy-1 and GAP-43. Fractions corresponding to detergent-resistant membranes (DRM) and to the soluble fraction (SF) are indicated. (C) Cholesterol detection in the sucrose gradient after Triton X-100 solubilization by means of TLC, as described under Experimental methods. Lane C corresponds to purified cholesterol used as a standard.

SNARE and SNARE-associated proteins are present in DRM

Once the raft separation was characterized, the distribution of SNARE and SNARE-associated proteins was analyzed (Fig. 2). The three proteins that form the SNARE complex (SNAP 25, syntaxin 1a/b, and synaptobrevin/VAMP2) showed a similar distribution after solubilization with 1% Triton, being present at significant levels in the insoluble fractions (Fig. 2). Munc18, synapsin 1, synaptophysin, and SV2 showed the same distribution, SV2 displaying a diffuse band due to its glycosylation state [19]. In contrast, rab3a and rab5 (two small monomeric GTPases that belong to the ras-related superfamily) were absent from DRM fractions. Finally, a high proportion of synaptotagmin I (approximately 40%, determined by densitometry of the bands) was found in DRM fractions (1–4), as assessed by using the monoclonal antibody 41.1 against synaptotagmin I. Subsequent analysis using two different polyclonal antibodies against isoforms I (against residues 1–19 from the rat synaptotagmin I) and II (against residues 1–17 from the rat synaptotagmin II) showed that approximately 40% of isoform I and 30% of isoform II, the two major neuronal isoforms, were associated with Triton X-100 rafts (Figs. 3A and B).

Effect of cholesterol depletion on DRM disruption and glutamate release from brain synaptosomes

Since it has been shown previously that the insolubility of lipid rafts in Triton depends on cholesterol, synaptosomes were treated with a combination of 0.5%

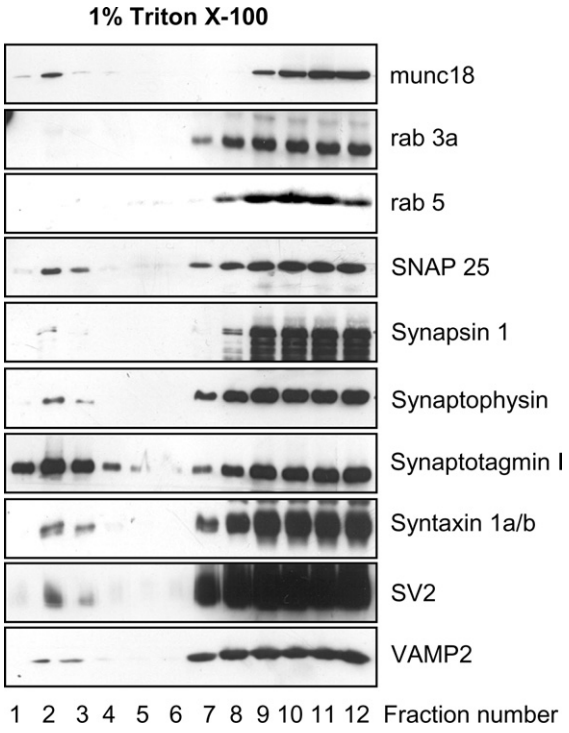

Fig. 2. Analysis of raft-associated proteins involved in membrane trafficking in rat brain synaptosomes. Synaptosomes were solubilized in 1% Triton X-100 and fractionated on a discontinuous sucrose gradient, as described under Experimental methods. Equal volumes of the recovered fractions were separated by SDS–PAGE and transferred to nitrocellulose for immunoblot analysis with antibodies against the indicated proteins. This analysis was repeated on four separate gradients with similar results. A representative result is shown. A monoclonal antibody (clone 41.1) against synaptotagmin I was used in these experiments.

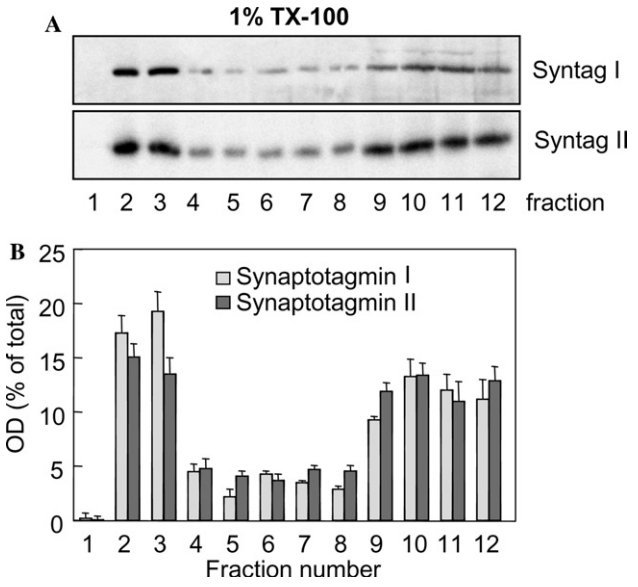

Fig. 3. Synaptotagmin isoforms I and II are enriched in DRM. (A) Synaptosomes were solubilized in 1% Triton X-100 and fractionated on a discontinuous sucrose gradient, as described under Experimental methods. Equal volumes of the recovered fractions were separated by SDS–PAGE and transferred to nitrocellulose for immunoblot analysis with antibodies against synaptotagmins I and II. (B) Graph showing the distribution of the isoforms along the gradient according to the optical density (OD) of the bands. Error bars indicate SD of three separate experiments. A representative result is shown in the Western blots.

saponin and 0.5% Triton X-100. Saponin belongs to a class of naturally occurring steroidal glycosides found in a variety of plants. This drug sequesters cholesterol, thus impeding its interaction with other membrane components [20]. Following treatment with saponin and Triton, all the proteins shown in Fig. 2 were effectively solubilized and no longer exhibited buoyancy in sucrose gradients. The gradient profiles of some of these proteins (munc18, SNAP 25, synaptotagmin I, syntaxin 1, and VAMP2) after saponin treatment are shown in Fig. 4A. Although the cholesterol-dependent insolubility of SNARE proteins strongly suggests that they are associated with cholesterol-dependent lipid rafts, incubation of synaptosomes with 50 mM methyl-β-cyclodextrin (MCDX), which extracts cholesterol from the membranes, for 15 min prior to solubilization with 1% Triton X-100 only resulted in a moderate loss of buoyancy of the proteins (Fig. 4B). In these conditions, only 10% of the initial cholesterol remained in synaptosomal membranes (data not shown), indicating that MCDX extraction of cholesterol is effective. This apparent lack of effect on buoyancy after MCDX treatment has also been described in other cases [21].

In order to assess the effect of cholesterol depletion on exocytosis, the potassium-dependent release of glutamate was studied. After stimulation of synaptosomes with 50 mM KCl, glutamate release was dramatically reduced in an MCDX concentration-dependent manner (Fig. 5A). To determine whether MCDX treatment affects steps prior to the interaction between the SV membrane and the plasma membrane, e.g., disruption of the role of calcium channels, 10 μM ionomycin (a calcium ionophore) was used instead of KCl to induce SV release (Fig. 5B). The abrogation by MCDX of glutamate release was also clear in the case of ionomycin, suggesting a principal role of cholesterol in the membrane-fusion machinery that leads to neurotransmitter release. Synaptosome integrity was also confirmed using a lactate dehydrogenase assay. The results of this assay indicated that MCDX treatment does not cause synaptosome disruption (data not shown). In summary, MCDX strongly impairs calcium-induced glutamate release without affecting the presence in lipid rafts of proteins involved in exocytosis. Disruption of the organization of these proteins within the lipid rafts could explain these apparently contradictory results.

SNARE complexes are present in DRM

The formation of synaptic core complexes is assumed to occur in a *trans* conformation at the interface between

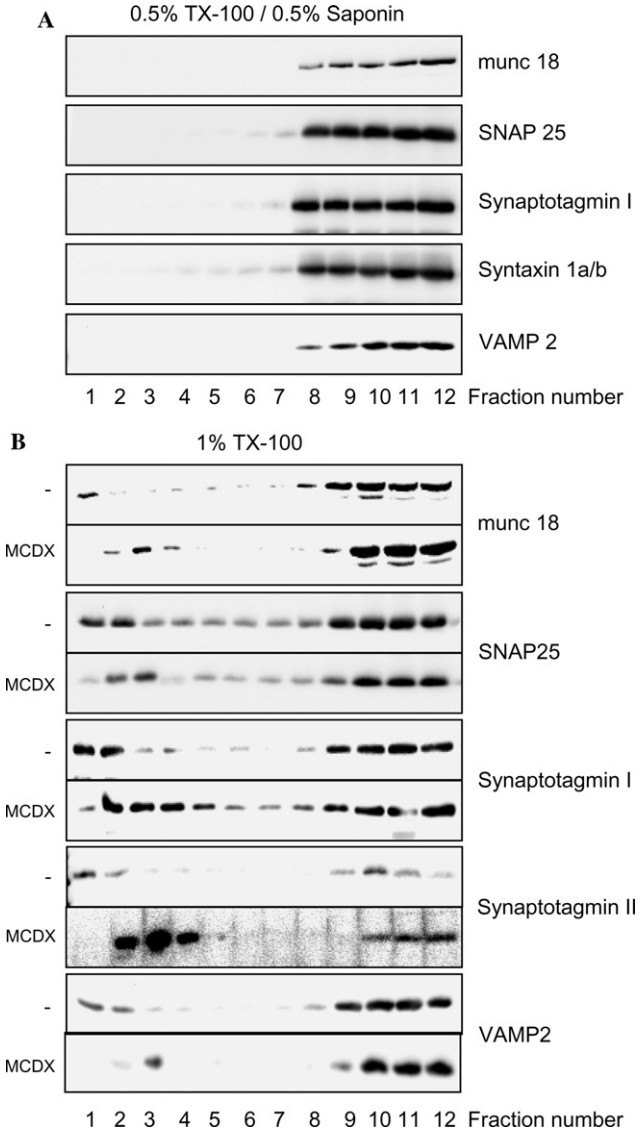

Fig. 4. Cholesterol dependence of raft association of SNARE and SNARE-associated proteins. (A) Synaptosomes were solubilized in 0.5% Triton + 0.5% saponin and fractionated on a discontinuous sucrose gradient, as described under Experimental methods. Equal volumes of the recovered fractions were separated by SDS–PAGE and transferred to nitrocellulose for immunoblot analysis with antibodies specific for munc18, SNAP 25, synaptotagmin I, syntaxin 1a/b, and VAMP2. (B) Synaptosomes were treated, where indicated, with 50 mM MCDX for 15 min prior to solubilization in 1% Triton X-100 and fractionation on a discontinuous sucrose gradient. Equal volumes of the recovered fractions were separated by SDS–PAGE and transferred to nitrocellulose for immunoblot analysis with antibodies specific for munc18, SNAP 25, synaptotagmins I and II, and VAMP2.

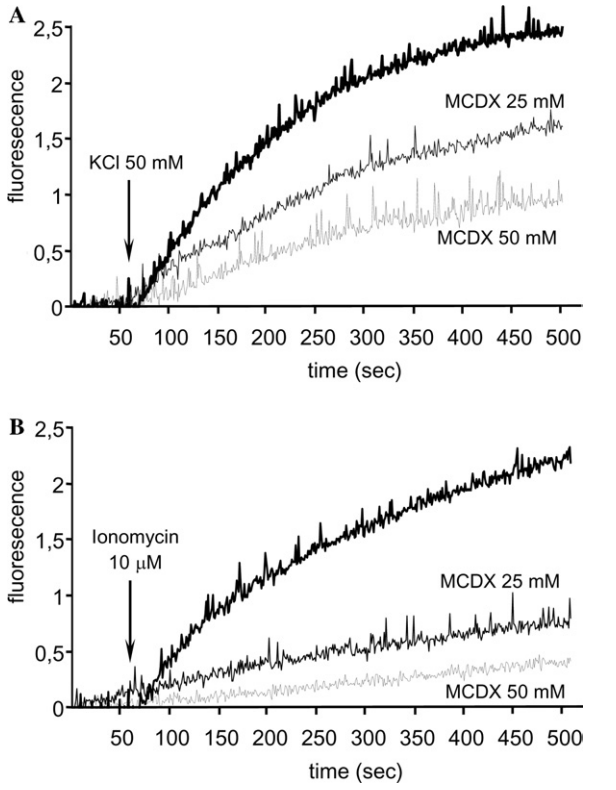

Fig. 5. Effect of cholesterol depletion on potassium-stimulated calcium-dependent glutamate release. (A) Synaptosomes were treated with or without MCDX (25 or 50 mM) for 15 min prior to induction of release with 50 mM KCl. Glutamate release into the medium was followed by fluorescence for 10 min, as indicated in Experimental methods. (B) Synaptosomes were treated with or without MCDX (25 or 50 mM) for 15 min prior to induction of release with the calcium ionophore ionomycin (10 μM). Glutamate release into the medium was followed by fluorescence for 10 min, as indicated under Experimental methods. The calcium dependence of the process was tested by addition of 1 mM EGTA, which totally abolished potassium-evoked glutamate release (not shown). Graphs shown are representative of three experiments, with similar results.

an SV and the presynaptic plasma membrane, and to constitute an essential step in SV fusion [22]. Ternary synaptic complexes display temperature-dependent resistance to dissociation with SDS, a property that can be used to assay complexes pre-existing in membranes [23,24]. Western blots, probed with antibodies against syntaxin 1 or SNAP 25, of fractions corresponding to DRM and SF from sucrose gradients after Triton X-100 solubilization revealed high molecular mass complexes (approximately 200, 80, and 50 kDa) that were stable in SDS at temperatures of up to 70 °C (Fig. 6). These complexes were present in Triton-DRM fractions as well as in soluble membranes, indicating that Triton-rafts are not preferential domains for SNARE complex formation in resting conditions. The complexes appearing at 80-kDa may be consistent with a binary complex containing a 2:1 ratio of syntaxin: SNAP 25 or with a ternary complex of syntaxin, SNAP 25, and VAMP2 in a 1:1:1 ratio [25]. Since potassium-triggered exocytosis is dependent on ternary SNARE complex formation [11], variations in the pattern of SNARE complexes after potassium treatment for 1 min were analyzed. The results obtained using an anti-syntaxin antibody did not show assembly or disassembly of complexes in any fraction after depolarization (data not shown). Incubation with an antibody against VAMP2 showed

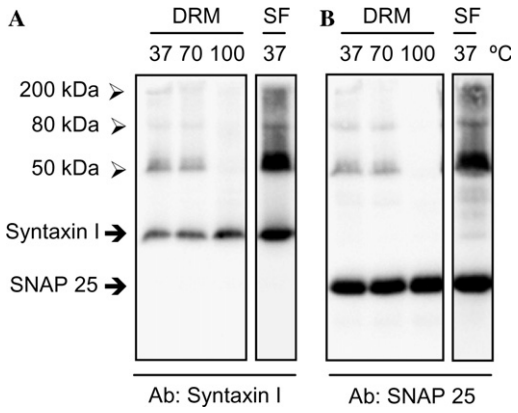

Fig. 6. SNARE complexes are also present in detergent-insoluble DRM. Aliquots from DRM or from the soluble fractions were incubated at the indicated temperatures in the presence of SDS. Western blots were prepared and probed with antibodies to syntaxin 1 and SNAP 25. The same SDS-resistant complexes are found in DRM and in SF, with an apparent M_r of approximately 200, 80, and 50 kDa. All these complexes contain both syntaxin 1 and SNAP 25.

a band corresponding to the monomeric protein, along with a band of higher molecular weight that most probably corresponds to VAMP2 homomeric complexes. This may be due to the fact that the epitopes recognized by the VAMP2 antibodies are masked when SNARE complexes are formed. Similarly, anti-synaptotagmin antibodies only gave a signal corresponding to monomeric synaptotagmin (results not shown), suggesting that its association with SNAREs is not SDS-resistant.

Discussion

To date, Triton X-100 has been the best-characterized and most commonly used detergent for the biochemical study of membrane microdomains and of the activities associated with them. The role of lipid rafts and cholesterol-rich domains in the regulation of exocytosis has been addressed in a number of studies [26,14,15], giving conflicting data. Two of these studies implicated lipid rafts, or similar cholesterol-rich membrane domains, in the regulation of exocytotic events in PC12 cells, since regulated exocytosis was impaired after cholesterol depletion using MCDX [14,15]. In addition, a recent paper [27] described the presence of SNARE proteins, synaptotagmin I, and Munc18 at low levels in DRM isolated with Triton X-100 from rat brain synaptosomes. Furthermore, it was shown in the same study that the P/Q-type calcium channels ($Ca_v2.1$) were exclusively present in these DRM, and that they interact with SNARE complexes and synaptotagmin I. This observation explains the impairment of depolarization-evoked calcium influx after cholesterol depletion by MCDX described in the literature, since this compound would alter the distribution and/or insertion of calcium and potassium channels in the membranes [27,28], as well as the association of the channels with their appropriate effector proteins, thus inhibiting channel activities [29]. Consequently, we used a calcium ionophore to bypass the possible effects of MCDX on calcium influx. Our results indicate that MCDX acts on a mechanism that occurs after calcium influx (Fig. 6B), which is consistent with observations using amperometric determinations in PC12 cells [15]. The proportion of synaptotagmins I and II present in Triton X-100-resistant DRM is higher than that of the SNARE components in the same fractions, suggesting that a large amount of synaptotagmin is not bound to the SNARE complexes. Both the synaptotagmin isoforms tested seem to be localized in the same kind of membrane domains, since the patterns are always identical. These observations are consistent with their localization in the same SV and their calcium-dependent oligomerization [30]. As stated above, SNARE complex formation is essential for membrane fusion during exocytosis [23], these complexes being stable even before evoked SV fusion [12]. The observation that the proportion of SNARE complexes found in DRM or in soluble fractions after Triton X-100 treatment correlates well with the proportion of monomeric SNARE proteins in the same fractions (Fig. 6), even under depolarizing conditions, is inconsistent with the view of lipid rafts as preferred sites for SNARE complex formation. This is not an obstacle for this limited pool of SNARE complexes present in lipid rafts to be important for exocytosis when this is triggered after a stimulus.

This study also shows an apparent discrepancy between the ineffectiveness of MCDX to extract the tested proteins from DRM and its dramatic inhibitory effect on glutamate release. To explain this discrepancy, the current models of raft structure proposed until now can be used. These can be classified according to three types: (i) homogeneous rafts with selective extraction of lipids; (ii) layered rafts, composed of concentric layers ranging from a well-ordered center to less ordered regions in contact with the bulk plasma membrane; and (iii) heterogeneous rafts with different lipid and protein composition [31]. According to the layered rafts model, MCDX will only be effective in the external region, being unable to extract the proteins resident in the core [32]. Consequently, the proteins that are unaffected by MCDX would remain in the core of the layered rafts, leaving their buoyancy properties unaltered. Nevertheless, the activity of the 'core proteins' would presumably require the presence of an intact outer layer, as would be the case during exocytosis, thereby offering an explanation for the apparent discrepancy between biochemical characterization and functional assays. This interpretation is supported by analysis of apical trafficking in HepG2 cells, in which cholesterol depletion induces missorting in the direct pathway without affecting raft insolubility [33].

Endocytosis is another process in which synaptotagmin is clearly involved [34]. Synaptotagmin has been described as binding to the clathrin-recruiting AP-2 adaptor complex at or near sites of exocytosis [35], thus participating in clathrin-mediated endocytosis and recycling of SV [36]. Lipid rafts have also been suggested to be involved in clathrin-independent endocytosis, since cholesterol depletion blocks uptake of many of the molecules reported to be internalized independently of clathrin-coated pits [37]. Similarly, nearly all of the molecules that are known to be internalized independently of clathrin are found in DRM [38]. Moreover, association of clathrin with lipid rafts has been described as necessary for B-cell antigen–receptor uptake [39]. In the same way, anthrax toxin triggers endocytosis of its receptors via a lipid raft-mediated, clathrin-dependent process [40], indicating that endocytosis via lipid rafts can be performed both in clathrin-dependent and -independent processes [41]. Thus, our observation of high amounts of synaptotagmin in DRM suggests that synaptotagmin may also participate in clathrin-independent endocytotic processes through lipid rafts. In summary, the results presented here support a direct involvement of cholesterol-rich membrane microdomains in the regulation of calcium-dependent exocytosis, in accordance with the observed presence of SNARE proteins and complexes in lipid rafts.

Acknowledgments

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