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Caveolin-3-anchored microdomains at the rabbit sarcoplasmic reticulum membranes ☆

Chunlei Li, Wenjuan Duan, Fuyu Yang, Xujia Zhang *

National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China

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Abstract

It is generally believed that sphingomyelin- and cholesterol-enriched microdomains can be isolated as detergent-resistant membranes (DRMs) from plasma membrane and organelle membranes. Here, we describe the isolation and characterization of microdomains from sarcoplasmic reticulum (SR) membranes. These SR-derived detergent-resistant membranes (SR-DRMs) enriched in sphingomyelin and cholesterol have a low buoyant density. Immunofluorescence microscopy of SR membranes shows the presence of caveolin-3 in the SR, known as a marker protein of caveolae at plasma membrane. We also demonstrated that significant amount of SERCAs together with caveolin-3 associates with SR-DRMs and are fully functional. Depletion of cholesterol caused the disruption of SR-DRMs.

Keywords: Sarcoplasmic reticulum; Ca^{2+}-ATPase; Caveolin-3; Microdomain

Low-density detergent-resistant membrane (DRM), which enriches cholesterol and sphingolipid, has been of great interests because it affects membrane protein functions and involves in the signal transduction and intracellular trafficking [1]. Recent observations suggest that DRMs are derived from at least two different types of microdomains at the plasma membrane, i.e., one has caveolae structure containing the marker proteins caveolin and the other does not [2]. However, microdomains are not likely to be restricted to the plasma membrane. Recently, microdomains from Golgi complexes have been characterized, which have a low buoyant density and are highly enriched in Golgi-derived sphingomyelin and cholesterol [3]. Moreover, lipid microdomains contribute to apoptosis-associated modifications of mitochondria in T-cells [4].

We have previously found that rabbit SR contains gangliosides, such as GM1 and GM3, and cholesterol as well [5]. The present study was therefore prompted to explore if DRMs exist at the SR membranes. Here, we reported the isolation and characterization of SR-derived detergent-resistant microdomains (SR-DRMs).

Moreover, the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is a major membrane protein at SR membranes and functions to pump Ca^{2+} from cytoplasm into the sarcoplasmic reticulum (SR) at the expense of ATP hydrolysis, maintaining the resting concentration of Ca^{2+} in cytoplasm. The activity of the ATPase is dependent on the chemical structure and physical phase of lipids of biological membranes where the enzyme resides [6,7]. It reported that the cholesterol and sphingolipids such as GM1, GM2, and GM3, the main components of DRMs, regulated the SERCA [8–10]. In the present study, we also studied the effects of SR-DRM on SERCAs. Our results show that the SERCA at SR-DRMs is fully functional.

☆ *Abbreviations:* SR, sarcoplasmic reticulum; DMSO, dimethyl sulfoxide; SR-DRM, SR-derived detergent-resistant membranes; SR-DSM, SR-derived detergent-soluble membranes; SERCA, sarco(endo) plasmic reticulum calcium ATPase; PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; LPC, lyso-phosphatidylcholine; Ch, cholesterol; GM1, monosialoganglioside-G_{M1}.

* Corresponding author. Fax: +86 10 6487 2026.
E-mail address: xjzhang@sun5.ibp.ac.cn (X. Zhang).

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Materials and methods

Materials. Cholera toxin subunit b conjugated to horseradish peroxidase (HRP) was from Sigma. The monoclonal antibody anti-SERCA1 was from CalBiochem Biosciences Inc. The monoclonal antibody anti-caveolin-3 was from BD Biosciences Pharmingen. The polyclonal antibody anti-caveolin-3 and HRP-conjugated goat-anti-mouse IgG secondary antibody were from Santa Cruz Biotechnology Inc. Anti-alkaline phosphatase polyclonal antibody was a gift from Dr. Pingsheng Liu. HRP-conjugated goat-anti-rabbit IgG secondary antibody and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat-anti-rabbit IgG secondary antibody was from Jackson Immunoresearch Laboratories Inc. Fluorescein isothiocyanate (FITC)-conjugated rabbit-anti-mouse IgG secondary antibody were from Rockland Immunochemicals Inc.

Phosphatidylcholine, phosphatidic acid, and cholesterol were from Avanti Polar Lipid Inc.

Preparation of highly purified sarcoplasmic reticulum. SR vesicles were prepared from rabbit skeletal muscle largely as described by Wang [5]. All procedures shown in Fig. 1A were carried out at 4 °C.

Isolation of low-density detergent-insoluble fractions from SR membranes. The low-density detergent-insoluble fractions from SR membranes were isolated using a flotation method [11]. Sixteen milligrams of SR vesicles (isolated as described above) was homogenized in 3 mL of a Triton X-100 (1%, v/v) solution in MN buffer (25 mM MES, 0.15 M NaCl (pH 6.5)) at 4 °C. After sonication of the suspension on ice, the homogenate was then adjusted to 45% sucrose by the addition of an equal volume of a 90% sucrose solution in MN buffer. The 4 mL homogenate was overlaid with 5 mL of a 35% sucrose and 3 mL of a 5% sucrose solution in MN buffer, and then centrifuged at 190,000*g* for 20 h. The gradient was fractionated into 12 fractions from top, 1 mL each.

Ca^{2+}-ATPase activity assay. ATPase activity was assayed using a coupled-enzyme method [12]. SR-DRMs were reconstituted into proteoliposomes containing a mixture of PC and PA (9:1, W/W) as described by Levy [13]. Ca^{2+} uptake was followed by dual-wavelength spectrophotometer using Arsenazo III to monitor the Ca^{2+} concentration [14].

Cholesterol depletion. SR membranes (3.98 mg of protein) were incubated in a total volume of 0.4 mL of SR suspension buffer with 100 mM methyl-β-cyclodextrin for 60 min at 30 °C. After centrifugation (100,000*g*, 30 min), the pellets were washed twice with 0.5 mL of SR suspension

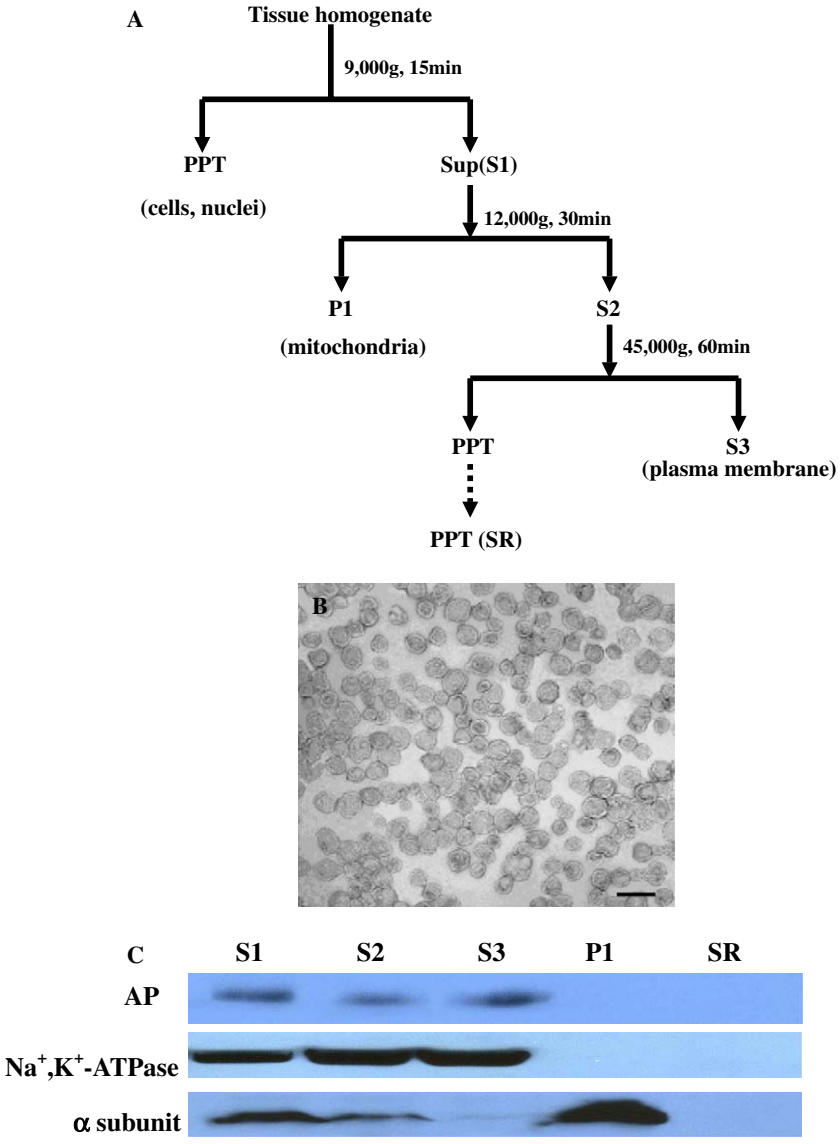

Fig. 1. Isolation of SR vesicles. (A) Isolation procedure of SR vesicles. (B) An electron micrograph of negatively stained SR vesicles. Bar = 0.4 μm. (C) Each fraction (100 μg/lane) was resolved by SDS–PAGE and immunoblotted with an anti-alkaline phosphatase and Na^+,K^+-ATPase for the detection of plasma membrane and α subunit of F_1F_o-ATPase for the detection of mitochondria.

buffer. The washed pellets were resuspended in 0.2 mL of SR suspension buffer and subjected to gradient fractionation as described above.

Lipid analysis. Samples (1 mL) were mixed with 1.2 mL chloroform and 1.2 mL methanol containing 2% acetic acid. After vortexing, the organic phase was collected and dried with nitrogen. The sample was redissolved in chloroform and spotted onto a HPTLC plate. To visualize sphingomyelin and cholesterol, the lipid samples were separated in a solvent system of chloroform/methanol/acetic acid/formic acid/H_2O (70:30:12:4:2, v/v/v/v/v) and hexane/isopropyl ether/acetic acid (65:35:2, v/v/v), respectively, and developed in vaporized I_2. Cholesterol was determined enzymatically using the cholesterol diagnostic kit. Total phospholipid content was determined according to the method of Rouser et al. [15].

Immunofluorescence microscopy. SR vesicles attached to coverslips were washed two times in PBS buffer (137 mM NaCl, 2.7 mM KCl, 9.8 mM Na_2HPO_4, and 1.7 mM KH_2PO_4, pH 7.4) and fixed for 20 min with 3.7% formaldehyde in PBS at room temperature. Afterwards, the vesicles were rinsed three times in PBS and then incubated in 5% normal rabbit serum in PBS for 40 min at 37 °C. Subsequently, the vesicles were incubated for 1 h at 37 °C with anti-SERCA1 monoclonal antibody (1:500) and/or anti-caveolin-3 polyclonal antibody (1:50) in PBS. The same procedure was also performed by using PBS instead of primary antibody as negative control, to ensure the specificity of the double-staining procedure and the specificity of fluorescent secondary antibodies. Afterwards, the vesicles were washed three times for 3 min in PBS and then incubated for 40 min at 37 °C with FITC-conjugated anti-mouse IgG (H+L) (1:400) and/or TRITC-conjugated anti-goat IgG (H+L) (1:200) antibodies. After washing the vesicles three times for 3 min in PBS, the coverslips were mounted with 50% glycerol in PBS. Images were acquired by a confocal laser scanning microscope (Olympus FV500, Japan) with the use of 488- and 543-lines of Ar–Kr and He–Ne lasers.

Results and discussion

Isolation of low-density detergent-resistant microdomains from SR membranes

SR membrane was isolated from rabbit muscle by previously established methods [5] (Fig. 1A). Electron microscopic examination revealed that the preparations were made of morphologically sealed vesicles with various sizes between 0.16 and 0.24 μm (Fig. 1B). The Western blot in Fig. 1C shows no detectable α subunit of F_1F_o-ATPase (a marker protein of mitochondria) in SR fraction. An antibody directed against alkaline phosphatase and Na^+,K^+–ATPase did not detect the presence of plasma membrane in the isolated SR vesicle fraction (Fig. 1C). We conclude that the SR vesicle fraction is largely free of contaminating organelles.

Triton X-100 insolubility at 4 °C is a commonly used criterion for membrane microdomains (referred to as detergent-resistant membranes, DRMs). On solubilization in Triton X-100, DRMs can be isolated as a low-density detergent-insoluble fraction resulting in flotation on a sucrose gradient [1,11]. The SR vesicles described above were therefore treated with 1% Triton X-100 and fractionated by sucrose density centrifugation. Since DRMs enrich sphingolipids and cholesterol, we first analyzed the ganglioside GM1, widely used as a DRM marker and known present at SR on a sucrose gradient. As shown in Fig. 2A, the

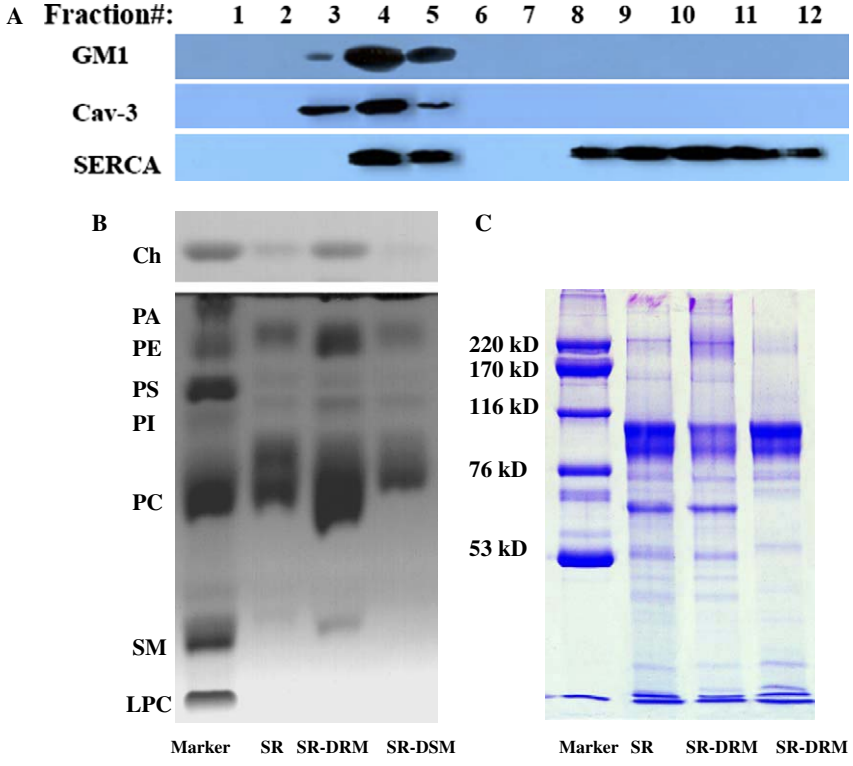

Fig. 2. Isolation of a low-density detergent-insoluble fraction from SR vesicles. (A) Flotation experiments were described in Materials and methods. Fractions (1 mL) were collected from top to bottom. An equal volume from each fraction was subjected to SDS–PAGE and Western blotting with the use of various antibodies as indicated. (B) Comparison of lipids among SR vesicles, SR-DRMs (fraction 4), and SR-DSMs (fraction 10). Lipids containing 100 μg of protein were separated on HPTLC plates and visualized as described under Materials and methods. (C) Protein composition of SR vesicles, SR-DRMs (fraction 4) and SR-DSMs (fraction 10). Protein (7.5 μg) was loaded on a 7.5% SDS–PAGE gel and stained with Coomassie blue.

GM1 is mainly floated to fractions 3–5 at the 5–35% interface, which is defined as SR-DRM. Caveolin-3 is a well-known marker for muscle-specific caveolae-derived complexes in the plasma membrane. However, SR localization of caveolin-3 has not been reported. It would be interesting to see if the caveolin-3 localizes at SR membranes and associates with SR-DRMs. Surprisingly, we found that SR membrane contains caveolin-3 (Fig. 2A). Moreover, the caveolin-3 is co-localized with GM1 in SR-DRM fractions (Fig. 2A).

The SR localization of caveolin-3 was further confirmed by immunostaining and confocal microscopy (Fig. 3). The green spots clearly showed the SR localization of SERCA. Immunostaining also showed the presence of caveolin-3 in the SR vesicles. Significantly, our findings indicated the considerable overlap in the immunostaining of the SERCA and caveolin-3. Because SERCA exclusively locates at SR membranes, our results demonstrate the co-localization of caveolin-3 and SERCA at SR membranes. Similarly, it has been reported that caveolin-1 is present in DRMs at the Golgi complexes [3].

The low density of SR-DRM is attributed to an enrichment of lipids (especially SM and cholesterol) in these complexes. The phospholipid to protein ratio of isolated SR-DRMs was determined and calculated to be 0.91 μmol Pi/mg protein (Table 1). For comparison, this ratio for SR membranes and SR-derived detergent-soluble microdomains (SR-DSMs) is on the average 0.55 and 0.22 μmol Pi/mg protein, respectively. This explains the low-density characteristics for SR-DRMs, running in the upper layers. The lipid composition of SR-DSMs was compared with those of SR membranes and SR-DSMs (Table 1 and Fig. 2B). This comparison clearly shows that SR-DRMs were enriched in SM, and cholesterol, while SR-DSMs were not. Additionally, 20% of PC, 88% of SM, and 73% of cholesterol of SR membranes were recovered in the SR-DRMs.

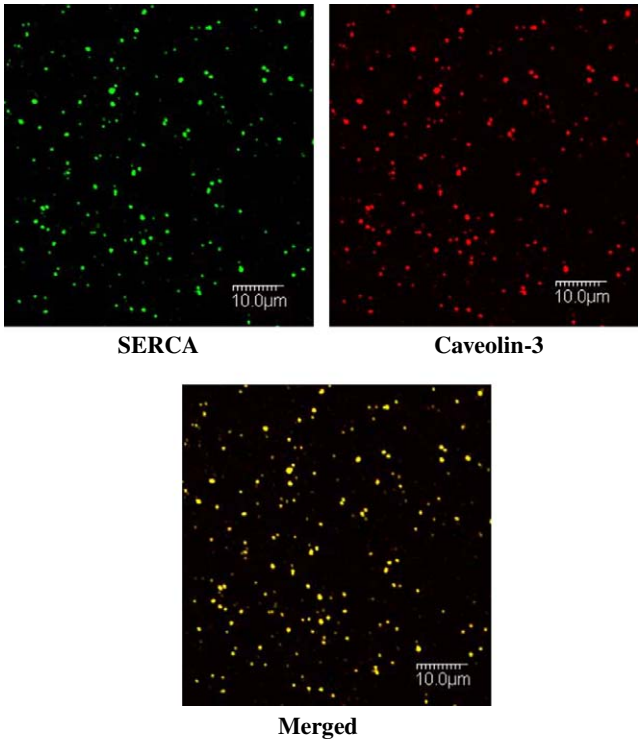

Fig. 3. Colocalization of SERCA and caveolin-3 in SR vesicles. SR vesicles were fixed and analyzed by double-immunofluorescence for SERCA (green) and Cav-3 (red) using confocal microscopy. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Table 1
Quantitation of lipid content of SR membranes, SR-DRMs, and SR-DSMs

	SR	SR-DRM	SR-DSM
PL* (mol Pi/mg protein)	0.55	0.91	0.22
PC (% of PL)	72	75	70
SM (% of PL)	3.6	16.6	Not detected
Ch (mol/mg Pr)	0.067	0.26	0.018
Ch/PL (mol/mol)	0.12	0.29	0.08

PL*, total phospholipids; PL, phospholipids without SM; lipids were measured and quantified as described in Materials and methods. SR-DRM and SR-DSM are from fractions 4 and 10, respectively. The data are expressed as mean values (±10%) from two different determinations.

The protein constituents in the SR-DRM preparation were also analyzed by SDS-PAGE and compared with those of SR membranes and the SR-DSMs (Fig. 2C). The protein composition of SR-DRMs shows relatively simple set of 9 proteins, comparing with total of 17 proteins in the SR membranes reflected by SDS–PAGE stained with Coomassie blue.

Association of SERCAs with SR-DRMs

DRM properties can affect functions of single proteins—particularly proteins with multiple membrane-spanning domains. For example, plasma membrane Ca^{2+}-ATPase was located at the caveolae of the plasma membranes [16,17], it was demonstrated that the caveolar ATPase was fully active [18]. Since SERCA is the major protein in the SR membrane, we tested the behavior of the enzyme on a sucrose gradient. The gradient was analyzed for the presence of SERCA (Fig. 2A). It can be seen that SERCA was evenly distributed along the gradient. Significant amount of SERCAs (~40%) is associated with SR-DRMs in view of the co-localization with GM1 and caveolin-3.

Showing the presence of SERCAs in SR-DRMs does not establish that this pool is catalytically competent, especially because the lipid composition of the SR-DRMs is known to be significantly different from that of the bulk SR membranes (Table 1 and Fig. 2B), and because SERCA activity is known to be dependent on the nature of phospholipids and the level of membrane cholesterol and sphingolipids [5,7,8]. It was important, therefore, to know whether the SERCAs detected in SR-DRM fractions are functional. We then measured the ATPase activities and Ca^{2+} uptake, and compared with those of the SR membranes. The results show that the specific activity (1.52 μmol Pi/min/mg) in SR-DRMs (fraction 4) is about

twofold higher than that in SR membranes (0.75 μmol Pi/min/mg) because SERCAs are concentrated and higher relative content in SR-DRMs. Moreover, the ATPase activity of SR-DRMs can be completely inhibited in the presence of 0.2 μM thapsigargin, the specific inhibitor of SERCA. Meanwhile, the reconstituted SERCA of SR-DRMs into proteoliposomes containing a mixture of phosphatidylcholine and phosphatidic acid is capable of transporting Ca^{2+}, indicating that SERCAs at SR-DRMs are fully functional.

Disruption of SR-DRMs by depletion of cholesterol

If a protein were found to be DRM-associated, treatment with cyclodextrin may be applied to test whether DRM association depends on cholesterol. SR membranes were then treated with 100 mM methyl-β-cyclodextrin (MβCD) for 60 min. As shown in Fig. 4, treatment led to disruption of SR-DRMs as judged by the redistribution of caveolin-3 and GM1 to, i.e., SR-DSMs. Similarly, SERCA was also redistributed, with the majority of the protein found in SR-DSMs. Our results suggest that cholesterol could be important to preserve SR-DRM integrity.

In the past few years many experimental observations support the membrane regionalization as a principle [19], in spite of controversy over the 'raft' hypothesis. Caveolin is a marker protein of caveolae, one of the best characterized types of microdomains. So the observation reported here, especially the presence of caveolin-3 at SR membranes and DRMs, may imply that microdomains also exist at SR membranes.

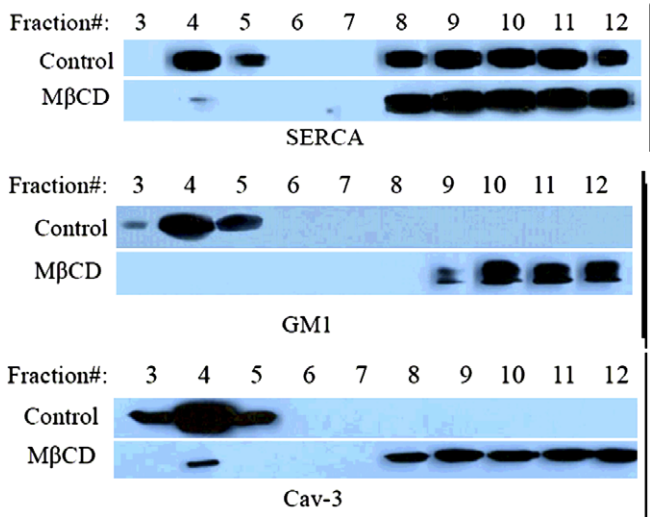

Fig. 4. Effects of MβCD on SERCA distribution. SR vesicles were treated with 100 mM methyl-β-cyclodextrin for 60 min at 30 °C. The treated and untreated vesicles were subjected to the Triton X-100 (1%) extraction and floated on sucrose gradients. Fractions 3–12 were analyzed by SDS–PAGE followed by immunoblotting for SERCA (top panel), GM1 (anti-CTxB, middle panel), and caveolin-3 (bottom panel). Untreated vesicles were as control.

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