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Detergent Resistant Microdomains at Cardiac Sarcoplasmic Reticulum Membranes*

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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 the isolation of microdomains from porcine cardiac sarcoplasmic reticulum (SR) membranes is described. These SR-derived detergent-resistant membranes (SR-DRMs) enriched in sphingomyelin and cholesterol have a low buoyant density. Both ganglioside GM1 and caveolin-3 in the SR, known as a marker protein of caveolae at plasma membrane are present in the SR-DRMs. It was demonstrated that significant amount of SERCAs together with caveolin-3 associates with SR-DRMs, and are fully functional. The results suggested that SR membranes like other biomembranes, such as the plasma membrane, contained microdomains.

Key words sarcoplasmic reticulum, Ca^{2+}-ATPase, microdomain

1 Introduction

Low-density detergent-resistant membrane (DRM), which enriches cholesterol and sphingolipid has been of great interests because it affects membrane protein functions and it is involved in the signal transduction and intracellular trafficking [1]. Recent observations suggest that DRMs are derived from at least two different types of microdomain 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].

The sarcoplasmic reticulum (SR) is an intracellular membrane system in cardiac cells, which plays a dominant role in cardiac excitation-contraction coupling cardiac contractility. A 1000-fold Ca^{2+}-gradient is maintained across the cardiac sarcoplasmic reticulum by the SR Ca^{2+}-ATPase (SERCA2a) [5]. We have previously found that rabbit SR from skeletal muscles contains gangliosides, such as GM1 and GM3, and cholesterol as well [6]. The present study was therefore prompted to explore if DRMs exist at the cardiac SR membranes. Here we reported the isolation of SR-derived detergent-resistant microdomains (SR-DRMs) from porcine heart.

2 Materials and methods

2.1 Materials

Anti-caveolin-3 and anti-Na^+, K^+-ATPase β2 were purchased from BD Transduction Laboratories. Cholera toxin subunit b conjugated to horseradish peroxidase (HRP) was from Sigma. TNAP(N-18) was purchased from Santa Cruz Biotechnology. Anti-SERCA2 ATPase Mouse mAb (IID8) was purchased from calbiochem.

2.2 Isolation of highly purified sarcoplasmic reticulum vesicles

Fresh porcine hearts were obtained from the local slaughter house. SR vesicles were prepared largely as

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described by[7]. All procedures shown in Figure 1a were carried out at 4℃. Briefly, ventricles were trimmed off fat and connective tissue and sliced into approximately 1 cm^3 pieces. The combined ventricular tissue (100～125 g) was homogenized in 500 ml of 30 mmol/L Tris (pH 7), 0.3 mol/L sucrose, 0.5 mmol/L DTT and 3 mmol/L NaN_3 using a PHILIPS mixer with four 15-s cycles of homogenization at full speed separated by an interval of 15 s. The homogenate was spun at 5 000 *g* for 10 min, and the supernatant (S1) was filtered through four layers of fine muslin, and then centrifuged at 23 400 *g* for 30 min. The resulting supernatant was centrifuged at 40 000 *g* for 60 min, and the pellets were homogenized in 125 ml of 20 mmol/L Tris maleate (pH 7.0), 0.3 mol/L sucrose, 0.6 mol/L KCl, 0.5 mmol/L DTT and 3 mmol/L NaN_3 using a Dounce homogenizer. After incubation for 25 min on ice, the suspension was centrifuged at 45 000 r/min for 60 min. The top loose pellet containing SR vesicles was collected by carefully adding 10 mmol/L Tris-HCl (pH 7.0), 0.3 mol/L sucrose, 1 mmol/L DTT, 1 mmol/L histidine and 0.2 mol/L KCl. After adjusting to 15～20 g/L, the cardiac SR vesicles were stored in liquid nitrogen and retained full activity for at least 3 months.

2.3 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[8]. 2 ml of SR vesicles (isolated as described above) were homogenized in 2 ml of a Triton X-100 (1%, *v*/*v*) solution in MN buffer (25 mmol/L MES, 0.15 mol/L NaCl (pH 6.5)) at 4℃. 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.

2.4 Cholesterol determination

Cholesterol was determined enzymatically using the cholesterol diagnostic kit. Total phospholipids content was determined according to method of Rouser *et al*[9].

2.5 Electron microscopy

SR vesicles dripped on carbon-coated grids were washed two times in double-distilled water. The specimens were negatively stained with uranyl acetate (1% *w*/*v*) and examined with a Philips Tecnai 20 electron microscope operated at 120 keV. The images were recorded on Lekai film at a magnification of 29 000 × and a defocus of –1 000 nm.

3 Results and discussion

SR membrane was isolated from porcine heart by previously established methods[7] (Figure 1a). Electron microscopic examination revealed that the preparations were made of morphologically sealed vesicles with various sizes (Figure 1b). An antibody directed against alkaline phosphatase and Na^+, K^+-ATPase did not detect the presence of plasma membrane in the isolated SR vesicle fraction (Figure 1c). We conclude that the SR vesicle fraction is largely free of contaminating organelles.

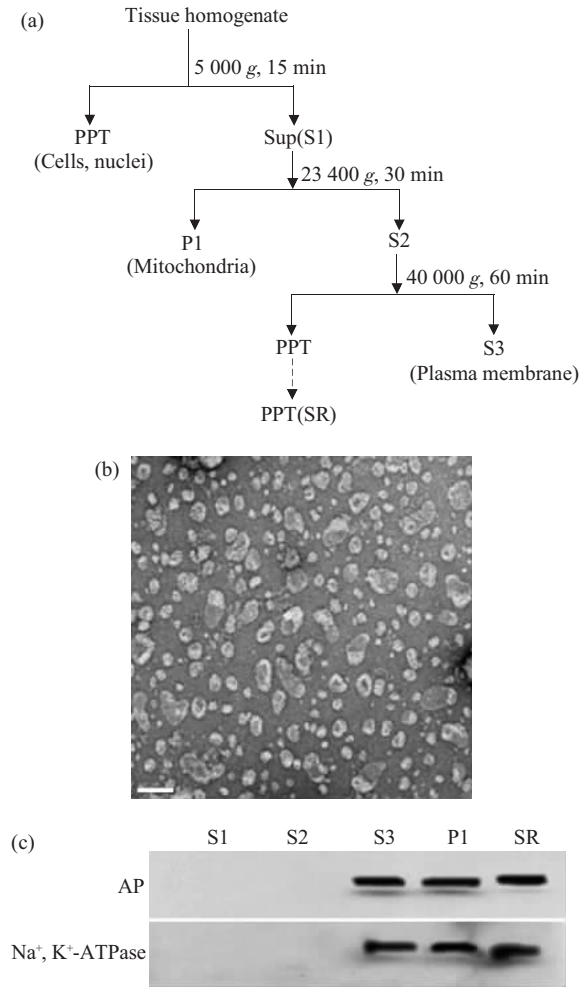

Fig. 1 Isolation of SR vesicles

(a) Isolation procedure of SR vesicles. (b) An electron micrograph of negatively stained SR vesicles. Bar = 100 nm. (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.

Triton X-100 insolubility at 4℃ is a commonly used criterion for membrane microdomains (referred to as detergent resistant membranes, DRMs) [10]. On solubilization in Triton X-100, DRMs can be isolated as a low-density detergent-insoluble fraction resulting in flotation on a sucrose gradient [1,8]. The SR vesicles described above were therefore treated with Triton X-100 and fractionated by sucrose density centrifugation. Figure 2 shows the distribution of total proteins in individual fractions on a sucrose gradient. It can be seen that the proteins are enriched in the SR-DRMs (fraction 3 ～5). The low density of SR-DRM should be attributed to an enrichment of lipids (especially SM and cholesterol) in these complexes. Then the SM and cholesterol were examined. It was found that both SM and cholesterol were enriched in SR-DRMs and were neglectable in SR-detergent soluble microdomains (SR-DSMs).

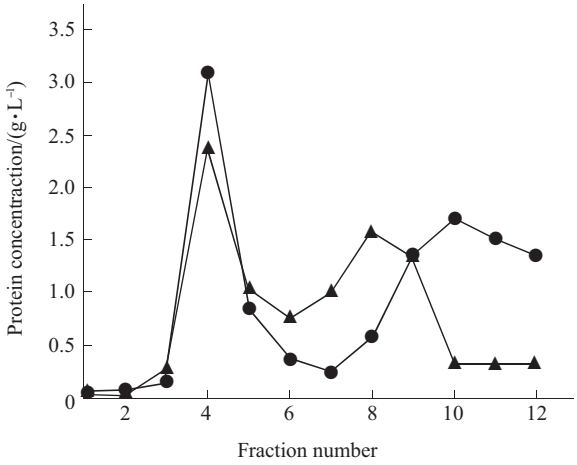

Fig. 2 Distribution of total protein in individual fractions (g/L)

SR vesicles were treated with 0.1% (▲—▲) and 1% (●—●) Triton X-100, respectively, and subjected to flotation experiments described in **"Materials and methods"**. Fractions (1 ml) were collected from top to bottom.

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 Figure 3a, the GM1 is mainly floated to fraction 3 and 4 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. Recently, caveolins were also found in organelle membranes, such as Golgi complexes[3]. So far, SR localization of caveolin-3 has not been reported. Therefore, we next examined the distribution of caveolin-3 at SR membrane. Surprisingly, we found that SR membrane contains caveolin-3. Moreover, the caveolin-3 is co-localized with GM1 in SR-DRM fractions (Figure 3b).

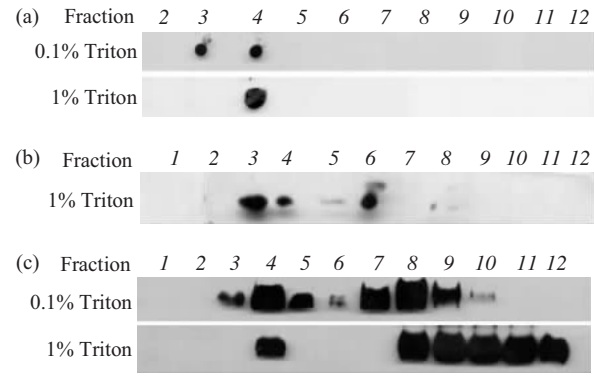

Fig. 3 Characterization of a low-density detergent-insoluble fraction from SR vesicles

An equal volume from each fraction was subjected to SDS-PAGE and Western blotting with the use of various antibodies. (a) GM1. (b) Caveolin-3. (c) SERCA2a.

SR Ca^{2+}-ATPase (SERCA2a), a marker of SR membranes, was distributed both in SR-DRMs and SR-DSMs (Figure 3c). For different detergent concentrations of Triton X-100, over 35% of SERCA2a was located in SR-DRMs treated with 0.1% Triton X-100 whereas less than 20% of SERCA2a was found in SR-DRMs treated with 1% Triton X-100. In cardiac tissue, the SERCA2a isoform facilitates the storage and distribution of Ca^{2+} in the SR, which is essential for cardiac relaxation. Our results clearly show that SERCA2a resides in at least two pools, one in SR-DRMs, and the other in SR-DSMs. Functional assay indicates that SERCA2as in both SR-DRMs and SR-DSMs are fully functional, *i.e.* capable of hydrolyzing ATP and transporting Ca^{2+}. Moreover, our preliminary results indicate that the SERCA2a in SR-DRMs shows higher activity, suggesting the functional heterogeneity of SERCAs at SR membranes.

In summary, SR membranes, Golgi complexes and mitochondria membranes containing DRMs suggest that DRMs exist at not only plasma membranes but also organelle membrane systems. Moreover, caveolin, a widely used marker for DRMs at the plasma membrane is now found not to be restricted to the plasma membrane, but associates with DRMs at organelle membranes, such as SR

membranes and Golgi complexes. The function of caveolin in organelle membranes remains to be determined. Our present study suggests that SERCA2as at SR membranes could reside at two pools, *i.e*. SR-DRMs and SR-DSMs. Possible functional differences of SRECA2as between in SR-DRMs and SR-DSMs should be further investigated.

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心肌肌浆网膜含有膜质微区结构 *

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摘要 细胞膜质微区 (microdomain) 是细胞膜上特殊的结构域，在细胞信号转导和物质运输过程中起着非常重要的作用. 绝大多数膜质微区来源于全细胞膜，即包括质膜和细胞器膜. 最新研究表明细胞器膜如高尔基体膜也有膜质微区，因此分离了猪心肌浆网膜的膜质微区. 首先获得了没有质膜污染的猪心肌浆网，用去污剂 Triton X-100 处理该肌浆网，获得了去污剂不溶的质膜微区(SR-DRM)，该微区富集胆固醇和鞘磷脂. 质膜微区的标记脂和蛋白质：神经节苷脂 GM1 和 Caveolin-3 也在该区富集. 同时还研究了心肌浆网 Ca^{2+}-ATPase (SERCA2a) 的分布，结果表明，相当数量的 SERCA2a 分布在膜质微区，并且有正常的生理功能. 上述研究结果表明，在心肌浆网膜上有膜质微区的存在，进一步证明膜质微区不仅存在于细胞质膜，也普遍存在于细胞器膜.

关键词 肌浆网，Ca^{2+}-ATPase，膜质微区
学科分类号 Q73

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